TNF gene expression in macrophage activation and endotoxin tolerance

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Nancy Ann-Marie Chow
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Abstract

TNF is an inflammatory cytokine that plays a critical role in the acute phase response to infection, and its dysregulation has been implicated in the pathology of several inflammatory and autoimmune disorders. *TNF* gene expression is regulated in a cell type- and inducer-specific manner that involves chromatin alterations at both the *TNF* promoter and distal DNase I hypersensitive (DH) sites within the *TNF/LT* locus. While the mechanisms underlying *TNF* gene activation in monocytes/macrophages and T cells have been studied intensively, the mechanisms of enhanced, repressed, and restored *TNF* gene expression in the context of classical macrophage activation and endotoxin tolerance remain largely unknown. We set out to understand how *TNF* gene expression is modulated during these biological processes by characterizing the chromatin environment of the *TNF/LT* locus.

In the context of classical macrophage activation, IFN-γ primes both circulating monocytes and tissue-resident macrophages so that, upon microbial recognition, there is an enhanced anti-microbial response; importantly, this results in enhanced *TNF* transcription. The mechanisms involved in priming are unclear; the IFN-γ-inducible transcription factors IRF1 and STAT1 have been implicated, but functional roles for these factors have not been determined. Here, we show in primary human monocyte-derived macrophages (MDMs) that IFN-γ poises the *TNF/LT* locus for enhanced *TNF* transcription by exposing the distal enhancer element hHS-8, and that the ability of this element to promote *TNF* gene expression in response to priming depends on its recruitment of IRF1. We also demonstrate that IFN-γ poises hHS-8 for enhancer function by promoting H3K27me3 enrichment, and that subsequent LPS stimulation triggers hHS-8 enhancer activation through demethylation of H3K27me3 followed by acetylation of H3K27. These experiments provide a mechanistic

explanation of how IFN-γ poises the *TNF/LT* locus for enhanced *TNF* transcription upon LPS stimulation, while at the same time providing potential targets for selective manipulation of TNF expression in primed macrophages.

In the context of endotoxin tolerance, repeated or prolonged exposure to LPS results in the reprogramming of monocytes and macrophages such that inflammatory responses, especially induction of TNF, are down-regulated; in this regard, the cell is considered tolerant or immunosuppressed. Notably, IFN-γ priming has been shown to abrogate endotoxin tolerance, thereby restoring responses to LPS stimulation. The mechanism of *TNF* gene repression during endotoxin tolerance and its restoration during IFN-γ-mediated abrogation remains unknown. Here, we have characterized the *TNF/LT* locus in endotoxin-tolerant monocytes and macrophages and found the *TNF* promoter to be nuclease accessible and enriched for H3K27me3 during repressed transcription. We have also found that IFN-γ priming restores *TNF* gene expression in an IRF1-independent manner, suggesting that IFN-γ primes non-tolerant and endotoxin-tolerant monocytes and macrophages by two distinct mechanisms; furthermore, we observed enrichment of H3K27ac at the *TNF* promoter and the appearance of a DH site ~4kb upstream of the *TNF* TSS in response to IFN-γ treatment of endotoxin-tolerant cells. These results suggest that chromatin modifications at the *TNF* promoter and distal DNA elements play an important role in IFN-γ-mediated abrogation of endotoxin tolerance.

Contents

Listing of figures

List of Tables

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Chapter 1

Introduction

1.1 Tumor Necrosis Factor (TNF)

The effects of TNF have been documented since the early 1890s when patients with malignant tumors were observed to undergo spontaneous tumor regression following acute bacterial infections [1]. In 1962, it was suggested that bacterial lipopolysaccharide (LPS)-induced tumor necrosis requires additional factors, as evidenced by the fact that LPS treatment did not kill tumor cells *in vitro* [2]. Finally, in 1975, Carswell and co-workers from Lloyd Old's group formally identified TNF in the serum of *Mycobacterium (M.) bovis* strain Bacillus Calmette-Guérin (BCG)-immunized mice treated with LPS [3]. They demonstrated that TNF-positive serum inhibited the growth of two tumor cell lines: Meth A sarcoma and L929. These results were later confirmed when recombinant TNF (rTNF) was shown to induce hemorrhagic necrosis of transplanted Meth A sarcomas in mice [4]. Soon after, rTNF was administered to tumor-bearing patients, but side effects were severe and reminiscent of symptoms associated with sepsis; patients were reported to experience headache, nausea, vomiting, fever, rigors, anorexia, and diarrhea—all signs of systemic bacterial infection [5]. These findings marked the beginning of a new stage in TNF research, with focus on TNF as primarily a mediator of host defense and inflammation.

1.1.1 TNF in infectious disease

TNF, in the context of infectious disease, elicits an array of cellular responses to bacterial, parasitic, and viral pathogens. It is a potent activator of endothelial cells, which line the surfaces of the skin, respiratory tract, gastrointestinal tract, oral cavity, genitourinary tract, and eye. During infection, TNF triggers endothelial cells to secrete soluble (e.g., IL-8, MCP-1, IP-10) [6–8] and cell surface molecules (e.g., ICAM-1, VCAM-1) [9, 10] that promote the accumulation of leukocytes and inflammatory mediators to sites of infection. By increasing local blood flow and vascular permeability, TNF consequently induces the four cardinal signs of inflammation: heat, pain, redness, and swelling [11]. In addition to its local effects, TNF is an endogenous pyrogen that induces fever via its effects on the hypothalamus [12]. Notably, TNF induces the expression of IL-1, which is a significantly more potent pyrogen than TNF [12, 13].

The creation of mice deficient for TNF signaling led to further insights into TNF biology. For example, studies using mice with a disruption in the *TNF* gene (*Tnf*-/-) have demonstrated a critical role for TNF in granuloma formation during *M. tuberculosis* infection. Granulomas are organized structures consisting of macrophages, epithelioid cells, foam cells, and multinucleated giant cells that are surrounded by a rim of lymphocytes; the granuloma's primary function is to contain the bacteria, thereby preventing their dissemination [14]. In *Tnf*-/- mice, T cells were found to migrate to the lung but failed to localize in infected alveoli and participate in granuloma formation. In addition, *Tnf*-/- mice succumbed to a low dose of aerosolized *M. tuberculosis* that did not cause mortality in wild-type mice [15].

Additional studies have demonstrated that mice deficient in the TNF receptor (*Tnfr1*-/-) were severely impaired in clearing the intracellular bacteria *Listeria (L.) monocytogenes* and *Salmonella typhimurium*, and readily succumbed to infection [16, 17]. TNF signaling is also critical for control of non-bacterial pathogens such as the intracellular parasite *Leishmania*

(L.) major; *Tnfr1*-/- mice inoculated with *L. major* were able to clear infection but failed to resolve lesions, suggesting a key role for TNF in healing lesions [18]. Finally, herpes simplex virus-1 (HSV-1) infection was lethal in *Tnfr1*-/- mice, and, compared to wild-type animals, *Tnfr1*-/- mice had significantly higher viral loads in the brain, suggesting a role for TNF signaling in controlling HSV-1 replication in the central nervous system [19]. In conclusion, multiple studies using mice deficient in TNF signaling have demonstrated the importance of TNF in host responses to bacterial, parasitic, and viral infections.

1.1.2 TNF dysregulation and its clinical implications

When over-expressed, TNF is a major mediator of the pathogenesis associated with several inflammatory and autoimmune disorders; these include rheumatoid arthritis (RA), inflammatory bowel disease (IBD), multiple sclerosis, plaque psoriasis, ankylosing spondylitis, and sepsis [20]. Although the pathogenic cause of most chronic inflammatory diseases is unknown, the classic inflammatory responses promoted by TNF are thought to account for chronic inflammatory pathology and tissue damage. For example, RA is characterized by lymphocyte activation in the peripheral blood, synovium, and synovial fluid; this causes inflammation in the joints and surrounding tissues. Examination of synovial fluid from symptomatic joints of RA patients showed the presence of active TNF [21], and stimulation of synovial fibroblasts with TNF resulted in the production of collagenase and prostaglandin E2, which are known to mediate bone resorption by osteoclasts [22]. In addition, chronic over-production of TNF in mutant mice lacking the TNF AU-rich elements ($TNF^{\Delta ARE}$) corresponds with inflammatory arthritis. AU-rich elements are sequences that help regulate TNF mRNA degradation and translational repression in hematopoietic and stromal cells [23]. Joints of $TNF^{\Delta ARE}$ mice exhibited features of chronic symmetrical inflammatory polyarthritis; indeed, both early and later stages of disease displayed characteristics comparable to human rheumatoid arthritis [24].

Anti-TNF therapy has been in clinical use since 1998 and has been overwhelmingly successful for the treatment of inflammatory diseases, particularly RA and a type of IBD known as Crohn's disease (CD). Two anti-TNF antibodies (infliximab and adalimumab) [25, 26] and a soluble TNF receptor fusion protein (etanercept) [27] bind and neutralize both soluble and membrane-bound TNF. These three biologics fuel a major part of the $20+ billion global market for TNF inhibitors and have been used to treat more than 2 million patients worldwide. Two novel TNF inhibitors, certolizumab pegol [28] and golimumab [29] have recently entered the market and have been approved for the treatment of CD and RA, respectively [30].

CD is the other major inflammatory disorder treated with TNF inhibitors; it is a type of IBD, along with ulcerative colitis [31]. CD is a chronic gastrointestinal disorder characterized by persistent disturbances between the commensal bacteria of the normal microbiome and the gut lumen, resulting in the induction of mucosal inflammation. TNF was first linked to the inflammatory responses of CD when higher levels of TNF were found in the mucosa and stools of CD patients as compared to those of healthy individuals [32]. In addition, TNF-positive macrophages were significantly increased in the lamina propria of CD intestinal specimens than that of control specimens [33]. These and other studies led to the 1998 US FDA approval of infliximab as the fist monoclonal antibody for CD treatment [34].

Although anti-TNF therapy has been extraordinarily successful in the last two decades, there are certainly adverse effects associated with global ablation of such a critical, non-redundant cytokine. Patients receiving TNF inhibitors have an increased risk for intracellular bacterial infections, including Mycobacteria [35], Listeria, Salmonella, and Legionella [36]. In fact, patients are required to undergo latent tuberculosis (TB) screening before initiating anti-TNF therapy; this is done as an effort to reduce the risk of TB reactivation [37].

In an effort to address the complications following systematic and continual TNF inhibition, recent studies have investigated the effects of selectively inhibiting TNF secreted from specific cell types [38]. The motivation is to primarily inhibit "pathogenic TNF" while sparing TNF that is essential for effective host defense. One study using mice deficient for TNF expression only in myeloid cells (i.e., monocytes, macrophages, neutrophils) showed that TNF produced by myeloid cells has a critical, non-redundant role in the early stages of *M. tuberculosis* infection but becomes redundant in the later stages of infection [39]. These mice were able to control chronic infection by recruiting activated, TNF-producing $CD4^+$ and $CD8^+$ T cells. In contrast, mice deficient for TNF expression only in $CD4^+$ and $CD8^+$ T cells were able to contain early *M. tuberculosis* infection but not persistent infection. Although granulomas were established, mice deficient for TNF in T cells were unable to efficiently recruit effector T cells to granulomas, thereby impairing the structural and functional integrity of granulomas during chronic infection. The authors conclude that TNF expressed from myeloid cells is primarily responsible for early immune function and that TNF expressed from T cells is essential to sustain protection against chronic TB infection. Thus, inhibition of TNF derived from T cells during latent *M. tuberculosis* infection may promote TB reactivation, suggesting that novel therapies for autoimmune diseases that are directed against myeloid-derived TNF would be preferable in latently infected TB patients.

In addition to directly suppressing TNF that is secreted from specific cellular sources, intracellular signaling mechanisms responsible for TNF production are also attractive targets for efforts aimed at developing more specific and less toxic anti-TNF therapies. In this regard, it is important to understand the mechanisms involved in regulation of TNF expression in various cell types at the level of both signal transduction pathways and chromatin, which was the overarching goal of this work.

1.2 Role of IFN-γ in macrophage function

A series of studies in the 1960s that examined *L. monocytogenes* infection in BCG-immunized mice introduced the theory that macrophage activation requires a humoral factor released by lymphocytes in the presence of a specific antigen [40]. This unknown factor was later named IFN-γ [41]. In the 1980s, it was established that IFN-γ and macrophage-activating factor (MAF) are one and the same when IFN-γ was shown to prime murine peritoneal macrophages for enhanced antiviral activity and LPS-induced tumor cell killing [42–44]. Since then, IFN-γ priming has been shown to enhance various monocyte and macrophage functions, including pathogen recognition, antigen presentation, microbial killing, receptor-mediated phagocytosis, and promotion of $CD4^+$ T cell differentiation toward a Th1 phenotype [45].

1.2.1 IFN-γ priming

Classical macrophage activation

Natural Killer (NK) cells, Th1 lymphocytes, and $CD8^+$ cytotoxic lymphocytes were originally thought to be the exclusive sources of IFN-γ; however, subsequent studies have demonstrated IFN-γ secretion from other cell types, including B cells, NKT cells, and antigen-presenting cells (APCs) [46–50]. In the early stages of bacterial infection, macrophages secrete chemokines and cytokines (i.e., IL-12 and IL-18) that both attract NK cells to the site of inflammation and induce their production of IFN-γ [51]. In addition, IL-12 and IL-18 drive Th1 differentiation of antigen-specific naïve T cells, which also go on to secrete IFN-γ [52]. IFN-γ subsequently converts resting macrophages into potent microbial killers, a process known as classical macrophage activation. Many TLR agonists, like LPS, are known to effectively activate macrophages without the assistance of IFN-γ [53], but the action of IFN-γ priming is essential for robust innate immunity characterized by potent macrophage

activity. Most notably, IFN-γ primes monocyte and macrophage responses to TLR agonists, thereby augmenting the transcriptional expression of critical inflammatory mediators such as TNF [54], IL-12 [55, 56], IL-6 [57], and NO [58]. In one study, relatively increased TNF mRNA levels were detected in LPS-stimulated primary human monocytes primed with IFN-γ as compared to unprimed cells [59]. The authors also showed that pretreatment of monocytes with IFN-γ for 2h was sufficient for augmented TNF mRNA levels, and that the effects of IFN-γ lasted for up to 40h after treatment. Similar findings were reported for IL-12 p35 mRNA levels in human PBMCs [60], IL-6 mRNA levels in the human monocytic cell line THP-1 [57], and NO mRNA levels in a mouse macrophage cell line [58]. A major goal of this work was to determine the mechanism responsible for IFN-γ priming, which, until now, has been unclear.

The importance of IFN-γ for innate immunity is evidenced by the clinical outcomes of patients lacking IFN-γ, its receptor, or its key signaling molecules. It has been observed in clinical studies that patients with disrupted IFN-γ signaling show increased susceptibility to mycobacterial infection; in particular, a deletion of nucleotide 131 in the gene for IFN-γ receptor 1 (*IFNGR1*) that results in a frame-shift and loss of IFNGR1 surface expression was found in a child with fatal idiopathic disseminated BCG infection [61]. In a similar study, a mutation (395A>C) in *IFNGR1* was associated with severe mycobacterial infections in four Maltese children [62]. This mutation led to decreased surface expression of IFNGR1 in PBMCs. The authors speculated that increased susceptibility to mycobacterial infection in the affected patients was primarily due to macrophage impairment, particularly impaired macrophage activation. Notably, PBMCs from the affected patients were stimulated with IFN-γ and LPS, and IFN-γ was unable to enhance TNF levels in patient PBMCs, as compared to control cells. Other children with disseminated atypical mycobacterial infection, who also had impaired IFN-γ production, exhibited defective TNF production in response to IFN-γ and LPS [63]. In conclusion, understanding the mechanisms responsible

for IFN-γ-induced macrophage activation has clinical implications for host defense against mycobacteria and a number of other intracellular organisms.

Endotoxin tolerance and its abrogation

Formally, endotoxin tolerance is the phenomenon in which immune cells, primarily monocytes and macrophages, transiently become hyporesponsive or "tolerant" upon repeated or prolonged exposure of LPS [64]. Physiologically, endotoxin tolerance is considered to be a negative feedback response that protects the host against uncontrolled inflammation, as seen in sepsis and severe tissue injury. One of the earliest documentations of endotoxin tolerance reported reduced fever responses in rabbits undergoing daily injections of typhoid vaccine [65–67]. This phenomenon of endotoxin tolerance was characterized as transient, evidenced by the finding that 3 weeks without injections rendered the rabbits responsive again. Decades later, induction of endotoxin tolerance in mice was shown to be a macrophage-mediated response [68]. Specifically, when LPS-resistant mice (later found to be defective for the LPS signaling receptor) were given LPS-sensitive macrophages from a wild-type mouse, they became susceptible to lethal challenges of LPS. In addition, when a group of these transplanted mice were first challenged with non-lethal doses of LPS and then subjected to a lethal dose of LPS, all mice survived, thereby demonstrating that macrophages are the primary mediators of endotoxin tolerance. Subsequent *in vitro* studies have shown induction of endotoxin tolerance in human monocytes and macrophages with reduced TNF expression as the primary readout [69–71].

Though endotoxin-tolerant monocytes and macrophages are often characterized as un- or hyporesponsive, these cells do adopt "active" mechanisms of control as well. In particular, gene expression of anti-inflammatory cytokines like IL-10, TGF-β, and IL-1RA was shown to be enhanced in monocytes from sepsis patients, as compared to those from healthy donors [72]. Another study observed increased internalization of *Escherichia coli* cells in

endotoxin-tolerant monocytes as compared to non-tolerant monocytes; additionally, higher levels of the surface receptor CD64, a well-known marker for phagocytosis, were found on endotoxin-tolerant monocytes as compared to non-tolerant monocytes [71]. Together, these results discredit the picture of endotoxin tolerance as macrophage "immunoparalysis".

Notably, IFN-γ has been shown to prevent the induction of endotoxin tolerance in human monocytes and macrophages. Co-treatment with recombinant IFN-γ during an initial challenge of LPS was shown to prevent endotoxin tolerance in Mono-Mac-6 cells, as evidenced by a restored induction of TNF mRNA levels upon a second challenge of LPS; notably, this was not observed in response to IFN-α and IFN-β priming [73]. Whether IFN-γ priming has a similar effect in both resting and endotoxin-tolerant monocytes is unknown. Indeed, the mechanisms responsible for endotoxin tolerance and IFN-γ-mediated abrogation remain unclear, and a major goal of the work described herein was to elucidate molecular mechanisms involved in these processes.

1.2.2 Crosstalk between IFN-γ and LPS signaling pathways

Components of the IFN-γ and LPS signaling pathways interact in various ways to enhance macrophage responses, as seen in resting monocytes and macrophages primed by IFN-γ [74]. The individual IFN-γ and LPS signaling pathways, and the interplay between components of the two signaling pathways, are described below.

IFN-γ signaling pathway

Signal transducer and activator of transcription-1 (STAT1) is the primary mediator of IFN-γ signaling [75]. IFN-γ binds to the IFN-γ receptor (IFNGR1), leading to the trans-phosphorylation and activation of receptor-associated janus kinase (JAK) 1 and JAK2 [76] (Figure 1.2.1). JAK1 then phosphorylates a subunit of IFNGR1 at tyrosine residue 440 (Y440) [78], thereby creating a docking site for STAT1. Via its SH2 domain, STAT1 binds

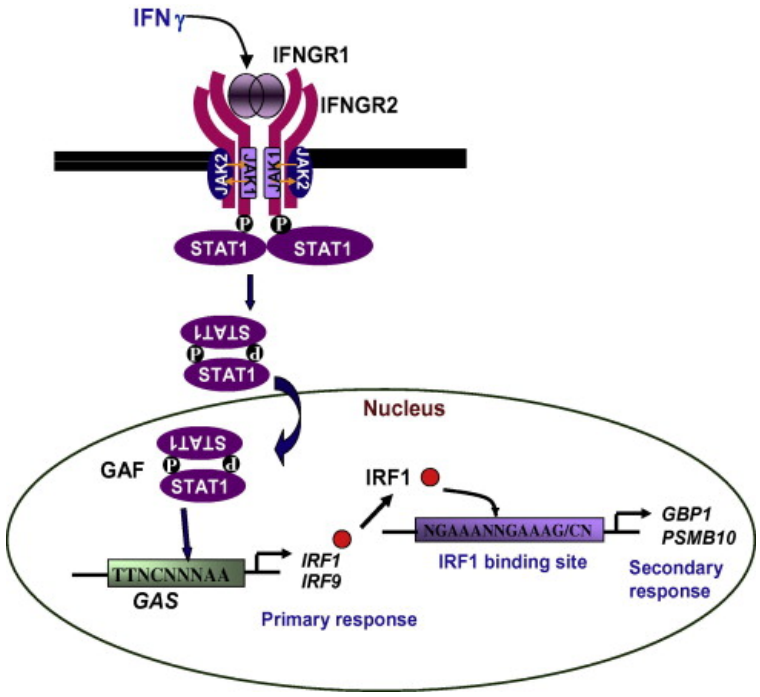


Figure 1.2.1: The IFN-γ signaling pathway. IFN-γ signaling activates the classical JAK/STAT pathway leading to STAT1 homodimerization and nuclear translocation. STAT1 binds to gamma-activated sequence (GAS) and activates the transcription of several primary response genes, including *IRF1*. Courtesy of (Saha, 2010) [77]

to the receptor and is then activated by phosphorylation of its tyrosine residue 701 (Y701) [79, 80]. Phosphorylated STAT1 homodimerizes, translocates to the nucleus, binds to a DNA element called gamma-activated sequence (*GAS*), and activates transcription of several primary response genes [77]. Notably, Stat1-/- and Ifng-/- mice have similar phenotypes in that they are non-responsive to both IFN-α and IFN-γ and are highly susceptible to microbial infection [75, 81].

One major primary response gene that is activated by IFN-γ signaling encodes Interferon Regulatory Factor 1 (IRF1). The IRF family consists of nine transcription factors that play pivotal roles in IFN-mediated immune responses [82]. IRF1 mediates the activation of several IFN-γ-inducible genes, including *NOS2*, *CASP1*, *COX2*, and *CIITA*. Interestingly, *Irf1-/-* mice exhibit impaired production of IL-12, which is an inducer of IFN-γ in T lymphocytes and NK cells [83]. Indeed, IRF1 recruitment to the *IL12A* promoter was shown

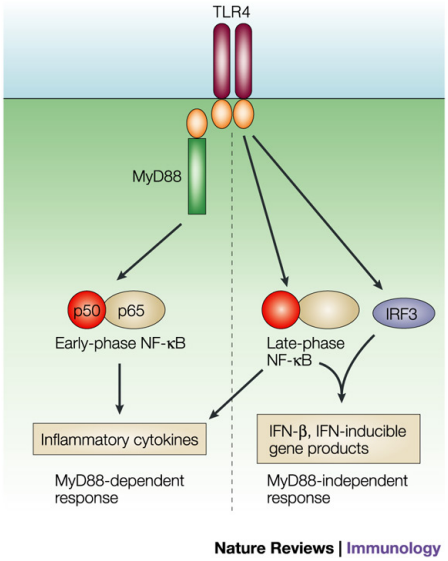


Figure 1.2.2: The TLR4/LPS signaling pathway. LPS signals through the TLR4 receptor and activates a MyD88-dependent or MyD88-independent pathway. The MyD88-dependent pathway involves NF-κB activation while the MyD88-independent pathway involves IRF3 activation and IFN-β expression. Courtesy of (Akira, 2004) [90]

to be necessary for enhanced IL-12 expression in monocytes primed by IFN-γ [84]. Thus, IFN-γ induces a positive feedback loop that is mediated by IRF1.

TLR4/LPS signaling pathway

With the help of several molecules, LPS binds to the extracellular domain of TLR4 and induces a signal transduction cascade that ultimately promotes (and suppresses) the expression of hundreds of genes [85]. Efficient recognition of LPS by TLR4 requires the co-receptor CD14 [86], LPS-binding protein (LBP) [87], and the accessory molecule MD-2 [88]. Signaling events downstream of TLR4 occur in either a MyD88-dependent or independent manner [89] (Figure 1.2.2). Importantly, production of TNF, IL-1β, and IL-6 in response to LPS was completely abolished in MyD88-deficient mice, highlighting the importance of this adaptor protein for expression of a subset of inflammatory genes [91]. Once MyD88 associates with TLR4, it recruits members of the IRAK family such as IRAK1, IRAK2, and IRAK4 [92]. IRAK4 phosphorylates IRAK1 [93], leading to their dissociation

from the receptor. IRAK1 binds TRAF6 [94] inducing the formation of a membrane-bound complex comprising IRAK1, TRAF6, TAK1, TAB1, and TAB2. TAK1 then activates IκB kinases (IKKs), which then form a complex, phosphorylate, and degrade IκB, an inhibitor of the transcription factor NF-κB. This results in the activation and nuclear translocation of NF-κB [95]. There are five subunits of the NF-κB family, but the subunits p50 and p65 associate to form the canonical NF-κB heterodimer, in which p50 binds to cognate DNA sequences and p65 activates transcription [96]. In addition to NF-κB, other important transcription factors like AP-1 and IRF5 are activated in a MyD88-dependent fashion [97].

Interestingly, there is a subset of LPS-inducible genes whose expression is not impaired in MyD88-deficient macrophages; these genes are induced via a MyD88-independent mechanism that requires IFN-β [98]. Upon LPS binding, TRAM is recruited to TLR4, resulting in the recruitment of TRIF [99, 100]. Subsequent events involving either RIP1 or TRAF6 lead to activation of NF-κB and AP-1 while events involving TBK1 lead to IRF3 activation by phosphorylation [90]. IRF3 and NF-κB assemble at the *IFNB* enhancer with other transcription factors and coactivators, thereby mediating the induction of *IFNB* gene expression. IFN-β, in turn, activates STAT1, which, as outlined above, leads to the induction of various inflammatory genes.

IFN-γ regulates TLR4/LPS signaling components

IFN-γ upregulates the expression of several TLR4/LPS signaling components. In human monocytes and macrophages, IFN-γ treatment was shown to augment TLR4 mRNA and TLR4 surface expression, as well as expression of crucial components of the receptor complex (i.e., MD-2 and MyD88) [101, 102]. In addition, LPS-induced phosphorylation of the downstream adaptor protein IRAK1 was significantly increased in endotoxin-tolerant monocytes treated with IFN-γ as compared to cells that were not treated with IFN-γ [103]. One study showed that treatment of monocytes with IFN-γ promoted IκB degradation and,

in turn, enhanced the DNA binding activity of NF-κB via EMSA analysis [104]; however, in another study, it was reported that IFN-γ does not affect IκB degradation but instead increases the expression of the NF-κB subunit p65 [105]. In any case, these combined data indicate that IFN-γ sensitizes the macrophage to LPS by upregulating crucial components for the receptor complex and by enhancing the activity of downstream signaling components.

In addition to enhancing TLR4/LPS signaling, IFN-γ suppresses feedback inhibitory mechanisms induced by TLR4/LPS signaling. IL-10 is a major anti-inflammatory cytokine that inhibits the activity of monocytes and macrophages, particularly the LPS-induced production of inflammatory cytokines [106]. IFN-γ has been shown to repress IL-10 production by inhibiting the transcription factor CREB, a critical component for *IL10* transcription [107]. Specifically, IFN-γ promotes phosphorylation and subsequent activation of glycogen synthase kinase 3β (GSK3β), a kinase that inhibits the function of AP-1 and CREB. The mechanism responsible for IFN-γ-mediated phosphorylation of GSK3β remains unclear.

Another mechanism by which IFN-γ inhibits anti-inflammatory responses to LPS stimulation involves the disruption of TGF-β signaling. Smad3 is an essential mediator of TGF-β signaling and induction of TGF-β-inducible genes. IFN-γ has been shown to impair Smad3 activation by (i) inhibiting its phosphorylation and subsequent nuclear translocation and (ii) inducing Smad7, an antagonistic Smad that prevents Smad3 interaction with the TGF-β receptor [108]. In conclusion, IFN-γ enhances innate immune activation by LPS via mechanisms that both promote inflammatory signaling processes and inhibit anti-inflammatory responses [109].

LPS regulates IFN-γ signaling components

As outlined above, IFN-γ augments LPS-induced signaling and the inflammatory response. At the same time, LPS has been shown to enhance the effects of IFN-γ stimulation

by increasing STAT1 phosphorylation at serine 727 (S727). Specifically, LPS stimulation in murine macropahges was shown to dramatically increase STAT1 phosphorylation at S727 at levels comparable to macrophages stimulated with IFN-γ [110, 111]. Again, STAT1 goes on to bind *GAS* and activate transcription of numerous IFN-γ-inducible genes. In addition to STAT1, LPS also activates the transcription factor PU.1 by phosphorylating its serine residue 148 (S148). PU.1 is a member of the Ets family of transcription factors and plays a pivotal role in macrophage maturation. Notably, PU.1 associates with IRF8, thereby forming a complex that binds *GAS* and enhances the expression of some IFN-γ-inducible genes [112].

1.3 Role of chromatin in inflammatory gene regulation

Identifying and characterizing signal transduction pathways is undoubtedly an essential process for defining regulatory mechanisms of the inflammatory response; however, recent studies have argued that chromatin status must be considered when explaining the selective nature of these mechanisms. In most instances, pathogen recognition by innate immune components results in the simultaneous transcriptional induction and inhibition of hundreds of genes whose chromatin environments are unique at each individual locus. Thus, it is critical to understand the many factors that comprise chromatin and regulate its conformational status in order to fully define the mechanisms responsible for inflammatory gene expression. When characterizing chromatin, several factors must be considered. These include DNA accessibility, transcription factor occupancy, histone modifications, DNA methylation, and higher-order organization.

1.3.1 Chromatin accessibility

In eukaryotes, DNA is packaged into chromatin, whose basic repeating unit is the nucleosome [113, 114]. Each nucleosome consists of ~146bp of DNA wrapped around an octamer

of four core histones [115]. When packaged into chromatin, DNA is relatively inaccessible to the TATA-binding protein and other factors that help mediate transcription; thus, nucleosomal DNA is considered to be repressive to gene expression. Importantly, regions of accessibility are created by the loss or remodeling of nucleosomes, which, in turn, provide docking sites for transcription factors and coregulators that bind DNA. Consequently, chromatin accessibility marks regions of DNA that function as regulatory elements such as promoters, enhancers, silencers, insulators, and locus control regions [116].

To detect regions of DNA accessibility in chromatin, investigators have traditionally relied on methods derived from the early finding that genomic regions that are accessible to transcription factors have a pronounced sensitivity to nuclease digestion, especially DNase I digestion [117, 118]. In fact, these regions are typically two orders of magnitude more sensitive to DNase I than nucleosomal DNA [116]. Pairing DNase I digestion with assays like Southern blotting, quantitative-PCR (qPCR), or DNA sequencing allows for the identification of regions that are accessible or hypersensitive to DNase I. These regions are termed DNase I hypersensitive (DH) sites and are usually markers of regulatory DNA elements [119, 120].

Within a given cell type, DH sites can either be constitutive or inducible [116]. Constitutive DH sites, as their name suggests, are independent of gene expression. Generally, these sites comprise promoter regions and are considered to be poised for transcriptional induction. In contrast, inducible DH sites appear prior to transcription and may persist long after the inducing agent is removed. One of the first inducible DH sites to be identified is in the *IL2* locus, which encodes IL-2, a pleiotropic cytokine produced by activated T cells [121]; human peripheral blood T cells were stimulated with mitogen, and an additional DH site appeared within the *IL2* promoter region. Since then, inducible DH sites have been found to not only be associated with transcriptional activation but also with transcriptional repression, demonstrating the diverse regulatory functions that individual DH sites can serve.

Recent studies have attempted to identify all DH sites in a given tissue or cell type with the aim of understanding cell type- or disease-specific regulatory networks. The Encyclopedia of DNA Elements (ENCODE) project has identified ~2.9 million unique, non-overlapping DH sites within the human genome [122]. A small proportion (~5%) of these sites localize to regions within 2.5kb of a transcriptional start site (TSS), while the majority of sites localize to regions greater than 2.5kb but within 250kb of a TSS. When classifying sites into either promoter or distal DH sites, the ENCODE authors designated any site found to be within 1kb upstream of a TSS as a promoter DH site. In addition, ~34% of these sites were specific to a single cell type while ~14% were detected in all 29 cell types examined. Interestingly, promoter DH sites generally were accessible across cell types in contrast to distal DH sites, which were found to be more limited to specific cell types. The differences in the degree of cell type specificity between promoter and distal DH sites highlight the importance of distal regulatory elements in the control of cell type-specific gene expression.

Notably, a vast majority of the distal DH sites exhibited chromatin features specific to enhancers. By definition, an enhancer is a small segment of DNA that augments transcription driven by its target promoter, which can be anywhere from several to hundreds of kilobases away [123]. For example, the limb bud enhancer for the mouse Sonic hedgehog (Shh) gene is located more than 1Mb from the *Shh* promoter [124]. Given the extreme variability among known enhancers in distance, orientation, and position relative to the target promoter, it is difficult to propose a single mechanism for enhancer function.

A representative example of enhancer complexity is found at the *IFNB* locus. Gene expression of IFN-β, an anti-viral cytokine primarily involved in innate immunity, utilizes a virus-inducible enhancer that is one of the best-characterized eukaryotic enhancers [125]. Proximal to the *IFNB* promoter, the enhancer is a 50bp element located ~100bp upstream of the *IFNB* TSS. The enhancer is subdivided into four positive regulatory domains (PRDs) designated PRDI, PRDII, PRDIII, and PRDIV; together, in response to appropriate stimula-

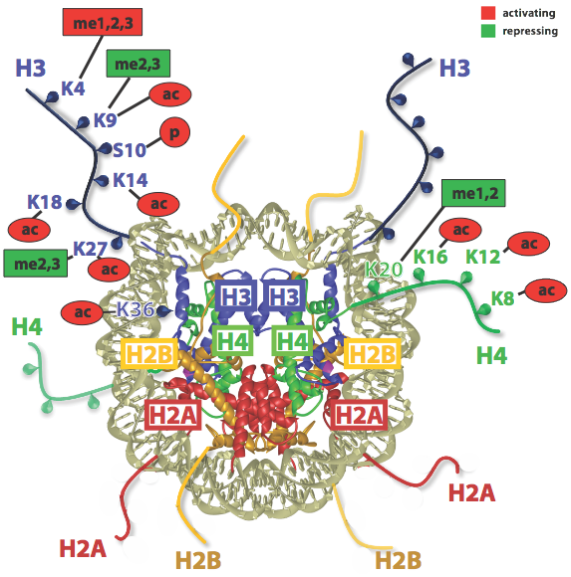


Figure 1.3.1: Structure of the canonical nucleosome. The histone octamer, illustrating the position of the N-terminal histone tails that are targets of post-translational modifications. Histones H3, H4, H2A, and H2B are shown in blue, green, yellow, and red, respectively. Diagram of a 2.8Å resolution structure [128, 129]. (Protein Data Bank code 1AOI) Kindly provided by Karolin Luger.

tion, these elements recruit a combination of factors that cooperatively bind and ultimately increase chromatin accessibility at the TATA box [126]. The *IFNB* enhancer provides one model of how chromatin accessibility is increased by a regulatory element; indeed, there are a variety of mechanisms that can be used to remodel chromatin.

1.3.2 Chromatin remodeling

Histone modifications

Considering the importance of chromatin accessibility for gene activation, many studies attempt to answer how chromatin is remodeled. Chromatin remodeling can be defined as any event that alters nuclease sensitivity of a region of chromatin [127]; one such event is the covalent modification of histones. There are five major families of histones: H1, H2A, H2B, H3, and H4. Typically, a nucleosome comprises two H2A/H2B heterodimers and an H3/H4 tetramer (Figure 1.3.1). Between each nucleosome core lies a stretch of linker DNA

ranging from 10 to 90bp that is either naked or bound by histone H1. Extending from the core domains are histone tails, which are often subjected to post-translational modifications like acetylation, methylation, ubiquitination, and phosphorylation; in fact, over 100 distinct histone modifications have been described [130].

Reversible histone acetylation was first proposed to be associated with transcriptional activity in eukaryotic cells in 1964 [131, 132]. This process locally relaxes chromatin by removing the positive charge on histone tails, thereby decreasing their affinity for the negatively charged phosphate groups of DNA. Consequently, histone acetylation promotes access to DNA for transcriptional regulatory factors. Histone acetyltransferases (HATs) transfer an acetyl group to the lysine residues of N-terminal histone tails, while histone deacetylases (HDACs) remove the acetyl group[133].

Histone acetylation has an important role in the activation of inflammatory genes. One such example is the gene *CSF2* encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), which is an inducible cytokine that functions to promote a Th1-biased immune response. Increased *CSF2* gene expression involves acetylation of lysine 8 and lysine 12 of histone 4 (H4K8ac and H4K12ac, respectively) at the human *CSF2* promoter [134]. Importantly, a specific stimulus can induce gene expression at different loci through distinct patterns of histone acetylation. For example, infection of human endothelial cells with *L. monocytogenes* was shown to upregulate both *IL8* and *IFNG* gene expression; however, increased H3ac and H4ac levels were only observed at the *IL8* promoter and not at the *IFNG* promoter [135].

Unlike acetylation, histone methylation does not chemically alter the charge of histone tails; rather, the effects of mono-, di-, and trimethylation of lysine and arginine residues are thought to indirectly affect gene regulation. Methylation of particular lysine residues is associated with gene activation (H3K4) and repression (H3K9 and H3K27) [136]. In the case of H3K9, trimethylation appears to enhance nucleosome stability and promote

heterochromatin formation by increasing the affinity of adapter proteins to the histone tail. Specifically, Swi6, a yeast ortholog of heterochromatin protein 1, forms a complex with H3K9me3-modified tails that ultimately facilitates heterochromatin spreading [137]. In the case of H3K27, trimethylation increases the affinity of the chromodomain of the Drosophila Polycomb protein for histone tails, which is implicated in the maintenance of gene silencing [138, 139]. The precise mechanisms of Polycomb-mediated repression remain unknown.

The balance of histone methylation is maintained by the interplay between histone methyltransferases and demethylases. EZH2, a subunit of Polycomb repressor complex 2, is a histone methyltransferase that has been shown to catalyze methylation of H3K27 [140–142]. JumonjiD3 (JMJD3) and UTX, two related histone demethylases specific to H3K27me3 [143–146], were shown to regulate inflammatory responses in the context of macrophage activation [147]. Inhibiting both JMJD3 and UTX activity in primary human macrophages resulted in reduced inflammatory cytokine levels upon LPS stimulation. In addition, JMJD3 and UTX inhibition prevented both the LPS-induced loss of H3K27me3 at, and the recruitment of RNA polymerase (Pol) II to, the *TNF* promoter [147].

Findings from recent studies have helped support the theory of "histone crosstalk" where different histone modifications "communicate" in elaborate combinations to remodel chromatin [148]. Within a population of numerous and distinct histone modifications, the order of adding and removing neighboring modifications has been shown to affect transcriptional readouts. For example, in the context of T cell activation, it has been found that H3K4me3 promotes H3K9ac and H4K16ac enrichment [149]. Specifically, when human $CD4^+$ T cells were treated with HDAC inhibitors, a subset of gene promoters that were enriched for H3K4me3 also became enriched for H3K9ac and H4K16ac; in contrast, promoters lacking H3K4me3 did not become enriched for H3K9ac or H4K16ac. Notably, the increase in histone acetylation corresponded to increased Pol II recruitment but not transcription initiation. Interestingly, a subunit essential for methylation of H3K4 has been shown to associate with

an acetyltransferase that recognizes H4K16 [150, 151]. These data indicate that H3K4me3 poises a subset of silent genes in resting T cells for histone acetylation, suggesting crosstalk among these histone modifications.

In addition to the covalent modification of histones, nucleosomes can be modified by deposition of histone variants in a replication-independent manner [152]. Histone variants are defined as non-allelic isoforms of canonical histones that differ in DNA sequence and, in some cases, amino acid sequence [153]. Replacement of canonical histones with histone variants can alter the packaging structure of nucleosomes, often leading to a less stable nucleosome [154]; indeed, histone variants H2A.Z and H3.3 have been shown to preferentially localize near transcriptionally active genes, particularly at promoters and enhancers [155–157]. For example, in one study IFN-β stimulation triggered extensive and prolonged H3.3 deposition at the 3'-coding regions of IFN-inducible genes like *Stat1* [158]. Specifically, IFN-β-induced transcription was significantly reduced in mouse embryonic fibroblasts where H3.3 was knocked down by shRNA. This data supports the finding that histone variants play a major role in chromatin remodeling and transcription.

Chromatin-remodeling enzymes

Chromatin-remodeling enzymes can be defined as transcriptional regulators that promote transcription factor binding by altering nucleosome packaging [127]. Importantly, they lack DNA-binding specificity and must be recruited by other mechanisms, these involve interactions with transcription factors, histone modifications, and non-coding RNAs [159, 160]. Generally, chromatin-remodeling enzymes fall into one of three categories: i) ATP-dependent remodeling enzymes; ii) histone-modifying enzymes; and iii) DNA-modifying enzymes [161].

The ATP-dependent SWI/SNF complex was one of the first chromatin-remodeling complexes to be analyzed in the context of inflammatory gene regulation. Upon ionomycin and

PMA stimulation, SWI/SNF was found to rapidly associate with chromatin, as evidenced by nuclear localization, in murine lymphocytes [162]. Subsequent studies have examined the role for SWI/SNF in macrophages activated by LPS and have found that SWI/SNF discriminates between secondary and primary response genes [163]. Specifically, secondary response genes and primary response genes with delayed kinetics were found to be SWI/SNF-dependent while rapidly induced primary response genes were found to be SWI/SNF-independent. Furthermore, the promoters of SWI/SNF-dependent genes were found to be relatively inaccessible prior to LPS stimulation as compared to the promoters of SWI/SNF-independent genes, which were nuclease accessible, suggesting a poised promoter state exists for rapidly induced primary genes.

In addition to activating gene expression, chromatin-remodeling enzymes are known to repress gene expression by promoting chromatin inaccessibility. For example, nuclear receptor complex (NCoR) is an essential component of chromatin-remodeling complexes that repress genes through a combination of HDAC and other enzymatic activities. During macrophage activation, dissociation of NCoR from a subset of inflammatory genes was shown to correlate with gene activation, suggesting NCoR and its accompanying complex function as active repressors of gene activation in resting macrophages [164]. Notably, the transcription factor c-Jun was found to be required for NCoR association with DNA, and LPS-induced phosphorylation of c-Jun led to disassociation of both c-Jun and the NCoR complex at activated genes [164]. Together, these data suggest a mechanism of LPS-induced gene activation that involves the removal of a repressive chromatin-remodeling complex.

Pioneer transcription factors

It is poorly understood how transcription factors, chromatin-remodeling enzymes, and histone variants are initially recruited to sites of repressed and/or inaccessible chromatin.

One possibility involves pioneer factors, a special class of transcription factors that can bind to relatively inaccessible DNA prior to the binding of other factors and gene activation [165]. Notably, the DNA binding domain of the pioneer factor FOXA1 was shown to resemble that of linker histone [166], which is known to bind nonspecifically to nucleosomes [167]. Pioneer factors are thought to either directly initiate chromatin remodeling events or to mediate the recruitment of chromatin remodeling factors to a particular region of DNA. A recent study examining the pioneer factor PU.1 in murine macrophages argued that pioneer factors may mark enhancers that will later become activated by developmental or environmental cues [168]. The authors looked at global PU.1 and p300 recruitment in both resting and LPS-treated murine macrophages and found PU.1 recruitment to be similar in both conditions while p300 recruitment was expectedly enriched at distal DNA elements upon LPS stimulation. Importantly, approximately half of the sites where inducible p300 recruitment occurred overlapped with sites of constitutive PU.1 binding. Thus, PU.1 appeared to constitutively mark distal DNA elements for LPS-induced p300 binding [168].

1.3.3 Chromatin looping

With the advent of chromosome conformation capture (3C) technology, the functional implications of chromatin looping have been more thoroughly examined. Chromatin looping has long been proposed as a mechanism underlying the function of eukaryotic enhancers, allowing for the enhancer to be brought into close proximity with its target promoter while simultaneously looping out intervening DNA [169]. Although DNA looping has been powerfully demonstrated by electron microscopy, microscopy does not allow for systematic studies of DNA topology at a high resolution [170]. Thus, 3C and its derivative assays have allowed for *in vivo* population-based analyses of nuclear organization at an unprecedented resolution.

In 3C-based assays, cells are fixed with formaldehyde, as is done in chromatin im-

munoprecipitation, to cross-link DNA/DNA, DNA/protein, and protein/protein interactions [171]. Chromatin is then digested, ligated under dilute conditions, and analyzed by PCR for chromatin interactions. Importantly, dilute conditions promote ligation of DNA ends that are in close proximity to one another, which in turn favors the ligation of junctions between cross-linked DNA fragments. The frequency of interactions is measured by the abundance of amplified PCR product and is compared to the random frequency with which various loci interact. Additional 3C-based assays exist that examine the role of a specific protein of choice or allow for genome-wide investigations of intra- and/or interchromosomal interactions [172].

A major finding using 3C technology is that, over an extent of 120kb, the promoters for the genes encoding T_H2 cytokines (i.e., IL-4, IL-5, and IL-13) are in close spatial proximity to one another [173]. This configuration was found in both $CD4^+$ T lymphocytes and NK cells, but was found to be absent in both B cells and fibroblasts. Essentially, a chromatin hub of interacting elements at the T_H2 cytokine locus forms in a cell type-specific manner. Another major study utilized 3C-based methods with data from the ENCODE consortium to comprehensively examine long-range interactions between TSSs and distal DH sites characterized as enhancers; in human cell lines, more than 1000 interactions were observed [174]. Importantly, significant correlations were found between promoter-enhancer interactions and gene expression. This is perhaps the most comprehensive study to have examined both the chromatin organization of regulatory elements and their functional relationships. In conclusion, 3C-based methods have greatly expanded our understanding of genome structure and mechanisms of action for distal regulatory elements such as enhancers.

1.4 *TNF/LT* locus and its transcriptional regulation

The *TNF* promoter and its distal DH sites have been shown to control transcriptional activation in response to multiple stimuli in a variety of cell types [175]. From a drug discovery

perspective, defining the mechanisms involved in the cell type-specific control of *TNF* gene expression in the context of the *TNF/LT* locus and its chromatin environment is critical for the identification of novel targets for selective therapeutic modulation of TNF.

1.4.1 *TNF/LT* locus

The *TNF/LT* locus comprises the *TNF*, *LTA*, and *LTB* genes; their coding regions occupy ~12kb on human chromosome 6p21 in a region of the major histocompatibility complex (MHC) between class I, B (*HLA-B*) and class II, DR (*HLA-DR*) [176]. In addition to the *TNF* promoter, the *TNF/LT* locus contains multiple DH sites [177–179]. Recent studies by our laboratory have identified multiple constitutive and inducible DH sites that exhibit both shared, and cell type specificity between primary human monocytes and $CD4^+$ T cells (unpublished results). The Goldfeld lab has labeled the distal DH sites based on their distance from the *TNF* TSS. For example, human hypersensitive site -12 (hHS-12) is ~12kb upstream of the *TNF* TSS.

hHS-12 is the most distal DH site in the *TNF/LT* locus and is predicted to be ~200 bp in length. It is located ~12kb upstream of the *TNF* TSS. Examination of the site in primary human monocytes and $CD4^+$ T cells showed the presence of hHS-12 in monocytes but not in $CD4^+$ T cells. The sequence is A/T rich (65% AT).

hHS-8 (also identified as DHS44500 [178]) is located ~8kb upstream of the *TNF* TSS and is predicted to be ~1.3kb in length. One study using ENCODE data that surveyed genome-wide DNase I sensitivity predicted that the average non-promoter DH site is ~200 to 300bp in length [122]; thus, hHS-8 is an unusually large non-promoter DH site. In contrast to hHS-12, hHS-8 was found in both human monocytes and $CD4^+$ T cells. In mice, hHS-8 is known as HSS-9 [177].

hHS-5 is located ~5kb upstream of the *TNF* TSS and is predicted to be ~300bp in length. Like hHS-12, hHS-5 is AT-rich (59% AT) and is monocyte-specific [180]. In mice, hHS-5

is known as HSS-7.

hHS-4 is located ~4 kb upstream of the *TNF* TSS and is predicted to be ~120bp in length. Importantly, hHS-4 is ~500bp upstream of the *LTA* TSS. Examination of monocytes and $CD4^+$ T cells showed hHS-4 to be present in $CD4^+$ T cells and not in monocytes, supporting the finding that LT-α is minimally expressed in resting and activated monocytes and is substantially expressed in activated $CD4^+$ T cells [181]. In mice, hHS-4 is known as HSS-4 [177].

hHS+6 is located ~6kb downstream of the *TNF* TSS and is predicted to be ~200bp in length. DHA analysis showed the site to be within the coding region of the *LTB* gene; however, it is more likely to be within a non-coding region proximal to the *LTB* gene. Similar to hHS-8, hHS+6 was found in both monocytes and $CD4^+$ T cells.

hHS+8 is located ~8kb downstream of the *TNF* TSS and is predicted to be ~130bp in length. Importantly, hHS+8 is ~1kb upstream of the *LTB* TSS. hHS+8 is similar to hHS-4 in that it was found to be present in $CD4^+$ T cells and not in monocytes, also supporting the finding that LT-β is minimally expressed in resting and activated monocytes and is substantially expressed in $CD4^+$ T cells [181–183].

Although most of the DH sites found in the human *TNF/LT* locus are also found within the mouse *Tnf/Lt* locus, there are a few differences. For instance, hHS-5 (murine HSS-7) is found within the human locus but is more prominent in the murine locus, suggesting that a smaller percentage of human cells as compared to mouse cells, on average, contain this site. Another major difference between the murine and human locus is that one site, HSS+3, is murine-specific. HSS+3 is ~600bp in length and is found immediately downstream of the *TNF* gene; it is present in both murine monocytes and $CD4^+$ T cells [177, 180].

1.4.2 *TNF* promoter and its transcriptional regulation

Originally defined as a product of monocytes and macrophages, TNF expression has been observed in $CD4^+$ T cells [46], B cells [184], NK cells [48], mast cells [49], dendritic cells [50], fibroblasts [185], and neurons [186]. In addition, *TNF* transcription is induced by a number of stimuli [175], including pathogens such as bacterial LPS, viruses, and parasites; mitogens; cytokines; and antigen binding by B cells and $CD4^+$ T cells. Indeed, as described below, *TNF* transcription is regulated in a cell type- and stimulus-specific manner.

At the level of the *TNF* promoter, specific sets of transcription factors and coactivators assemble to form nucleoprotein complexes known as enhanceosomes [187–189]. Both protein-protein interactions and the conformational changes induced upon initial protein-DNA interactions facilitate the cooperative binding of additional factors to adjacent and overlapping sites. Additionally, coactivators that associate with multiple transcription factors can serve as "scaffold" proteins, thereby encouraging enhanceosome formation [190]. The first enhanceosome was described during the study of *IFNB* gene activation, and was shown to include at least three distinct transcription factors and the high mobility group protein HMG I(Y), which function in synergy to drive transcription [191, 192]. Each of the transcription factors recruited to the *IFNB* promoter has been shown to interact with CBP/p300, a histone acetyltransferase known to acetylate histones and non-histone proteins. In addition to acetylating histones within the *IFNB* promoter, CBP/p300 is thought to function as a scaffold protein, facilitating enhanceosome assembly [193].

Enhanceosome formation at the *TNF* promoter is particularly complex by comparison in that it has been shown to occur in a cell type- and inducer-specific fashion. The proximal *TNF* promoter maps to a highly conserved region ~200bp proximal to the *TNF* TSS, and is sufficient for transcriptional activation in response to multiple stimuli [189, 194–196]. Within the *TNF* promoter, there is a TATA box and multiple transcription factor binding sites, including six nuclear factor of activated T cells (NFAT) sites, four Ets/Elk-1 sites, two

Sp1 sites, an Egr site, and an ATF-2/c-Jun site [175] (Figure 1.4.1). In T cells, enhanceosome formation was found to be inducer-specific; ionomycin, which increases intracellular $Ca2^{+}$ levels, was shown to promote NFAT binding to all six NFAT sites whereas Sendai virus infection was shown to promote less NFAT binding than ionomycin [187]. Differential NFAT binding at the promoter was partly due to differences in regional NFAT concentration, as more NFAT translocates into the nucleus upon ionomycin stimulation than after viral infection [187]. In addition, Sp1 binding was observed to a much larger extent at the -55NFAT/Sp1 site after Sendai virus infection as compared to ionomycin stimulation, and was shown to synergize with NFAT and ATF-2/c-Jun [198]. Importantly, the -55NFAT/Sp1 site is a fairly weak Sp1 site; thus Sp1 is outcompeted in scenarios of high NFAT levels like ionomycin stimulation. In conclusion, *TNF* transcription in T cells provides a striking example of stimulus-specific gene regulation that encompasses both regional concentration of specific transcription factors and affinities of binding sites.

In the case of monocytes and macrophages, LPS stimulation results in the formation of an enhanceosome that is distinct from that found in T cells in response to ionomycin treatment or Sendai virus infection. In particular, two Sp1 sites, four Ets/Elk-1 sites, an ATF-2/c-Jun site, and an Egr binding site were found to be critical for LPS induction of *TNF* transcription in macrophages [189]. Notably, Ets and Elk-1 proteins were found in place of NFAT at the *TNF* promoter upon LPS stimulation. Thus, the presence of overlapping DNA motifs that can serve as both NFAT and Ets/Elk-1 binding sites allows for a broad spectrum of distinct enhanceosomes to form at the *TNF* promoter depending on cell type and the signaling pathways triggered. Interestingly, *M. tuberculosis* induction of *TNF* transcription in macrophages involves an enhanceosome of similar composition to that of LPS induction [195].

In the case of LPS stimulation in monocytes and macrophages, some initial studies suggested a role for the transcription factor NF-κB, which is now known to play a key role in

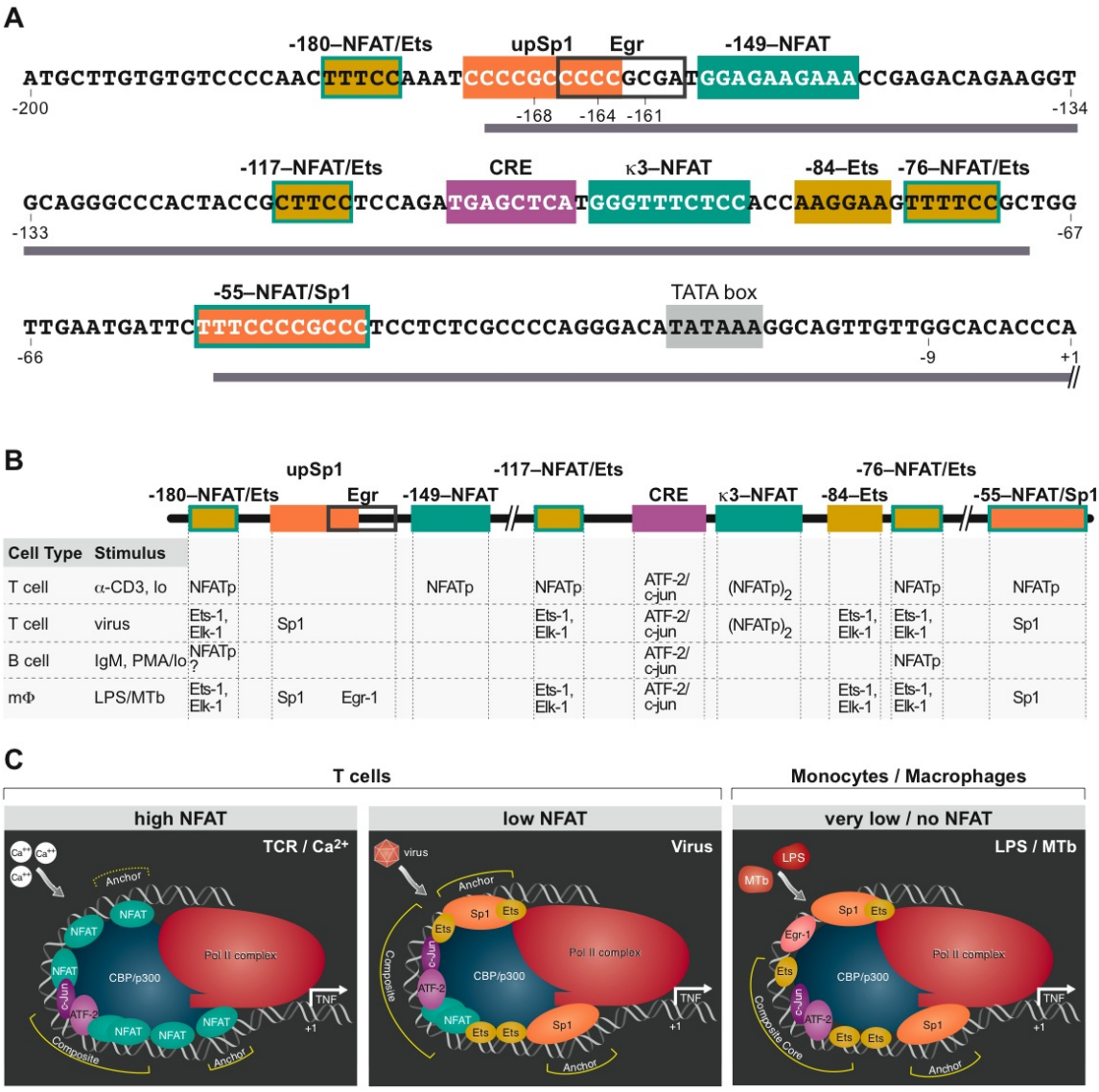


Figure 1.4.1: Cell type- and inducer-specific enhanceosome formation at the proximal *TNF* promoter. (a) Sequence of human proximal *TNF* promoter showing the positions of transcription factor binding sites. (b) Transcription factors that bind at sites in the indicated cell types and in response to the indicated stimuli. (c) Model of inducer-specific enhanceosome formation at the proximal *TNF* promoter in T cells and monocytes. [175, 195, 197].

TLR signaling. Multiple NF-κB sites lie upstream of the *TNF* TSS, but these are generally not conserved between mice and humans and lie outside of the proximal promoter [196, 199, 200]. The one NF-κB-like site present in the proximal promoter, originally designated κ3 [196], is bound by NFAT proteins and functions as a calcineurin-dependent composite element within the adjacent CRE site. This site binds ATF-2/c-Jun heterodimer, and is required for *TNF* transcription in response to multiple stimuli [187, 189, 194, 195, 198, 201–206]. Through EMSA and quantitative DNase I footprinting analysis, recombinant p50/p65 was shown not to bind specifically to κ3 or any other site within the proximal *TNF* promoter. In addition, assays targeting NF-κB activity in monocytes and macrophages indicated a post-transcriptional induction of NF-κB in response to LPS for maintaining TNF mRNA levels in these cells, rather than a direct role in initiation of *TNF* transcription [207].

As stated previously, a primary function of transcription factors is to promote the recruitment of nucleosome remodeling enzymes to regulatory regions, thereby promoting chromatin accessibility and subsequent binding of RNA Pol II. Importantly, CBP/p300 has been shown to play a major role in *TNF* transcription in both macrophages and T cells [188, 189, 195]. Inhibition of CBP/p300 impairs *TNF* transcription induced by LPS in murine macrophages [188]. Strikingly, examination of splenic T cells from $Cbp^{+/-}$ mice showed that induction of *TNF* transcription by TCR engagement was dramatically reduced as compared to what is found in cells isolated from wild-type or $p300^{+/-}$ mice, indicating a novel inducer-specific role for CBP in TNF enhanceosome formation in activated T cells [188]. This conclusion was indeed supported by later findings in conditional knockout mice [208]. Together, these studies demonstrate the importance for CBP/p300 in *TNF* transcriptional activation and support a model where CBP/p300 acts as a "scaffold" protein during enhanceosome formation at the *TNF* promoter.

1.4.3 Distal regulatory elements at the *TNF/LT* locus

As stated previously, multiple DH sites distal to the *TNF* promoter lie within the *TNF/LT* locus. Importantly, many of the identified DH sites coincide with regions of highly conserved DNA, which suggests regulatory function. From an evolutionary perspective, functionally relevant sequences are typically subjected to negative selection, whereas non-functional sequences are subject to genetic drift and become increasingly different between species with increasing phylogenetic distance [209]. Thus, highly conserved non-coding DNA sequences are predicted to be regulatory elements. Extending the findings of conserved functional noncoding DNA sequences in the *TNF* promoter [197, 199, 210], other DH sites in the *TNF/LT* locus, including hHS-8, were found to be strongly conserved between mice and humans, as well as a number of other species [178].

DH sites within the murine *Tnf/Lt* locus have been shown to undergo inducible intrachromosomal interactions while functioning as enhancers. Increased levels of H3ac and H4ac were found at both HSS-9 and HSS+3 in murine T cells in response to anti-CD3/CD28 activation, suggesting inducible chromatin remodeling at these distal sites in the murine *Tnf/Lt* locus [177]. In addition, both HSS-9 and HSS+3 were shown to enhance *Tnf* promoter activity in a murine T cell line in an NFAT-dependent manner. Finally, it was demonstrated through 3C analysis that an inducible intrachromosomal interaction between the *Tnf* promoter, HSS-9, and HSS+3 occurred upon activation of murine T cells. Thus, the interplay of histone modifications, transcription factor binding, and intrachromosomal interactions orchestrate inducible enhancer function at the murine *Tnf/Lt* locus, allowing for rapid and potent activation of this critical immunological factor during innate immune responses.

1.5 Summary of Aims

Macrophages are fundamental cellular components of the innate immune response. Although the genetic programs and signal transduction pathways that contribute to macrophage activity in response to microbial infection have been thoroughly investigated, much less is known about the chromatin-remodeling factors and related chromatin environment that orchestrate these cellular processes. The overarching goal of this thesis project was to identify and dissect mechanisms of chromatin remodeling that control classical macrophage activation and endotoxin tolerance. To achieve this goal we used the *TNF/LT* locus as our model system. This locus encodes TNF, the archetypical inflammatory cytokine whose enhancement and repression is representative of classical macrophage activation and endotoxin tolerance, respectively.

Our first aim was to test the hypothesis that potent *TNF* gene expression in response to classical macrophage activation, where a priming signal by IFN-γ results in augmentation of *TNF* gene expression upon LPS stimulation, is regulated, at least in part, by IFN-γ-mediated chromatin modifications at the *TNF/LT* locus. Findings from experiments designed to address this hypothesis are described in Chapter 2. Our second aim was to test the hypothesis that chromatin modifications at the *TNF/LT* locus are also critical for the modulation of *TNF* gene expression during the induction of endotoxin tolerance in macrophages, and that the ability of IFN-γ to restore LPS-induced *TNF* gene expression in endotoxin-tolerant macrophages depends on similar chromatin changes as those induced by IFN-γ at the *TNF/LT* locus during macrophage priming. In Chapter 3, we present findings from experiments performed to address this hypothesis.

A diverse array of experimental techniques were employed to achieve the overall goal of this project, including DNase I hypersensitivity analysis, DNase I footprinting, chromatin immunoprecipitation (ChIP), and genetic knockdown and mouse knockout systems. The

experiments were performed, when possible, using primary human macrophages in order to obtain data of clinical relevance. The findings described in this dissertation provide novel insights into mechanisms of chromatin remodeling at play during macrophage priming, induction of endotoxin tolerance, and abrogation of endotoxin tolerance. We expect these findings to be broadly applicable to other primary response genes that are similarly affected by these biological processes. In addition, we anticipate that the findings described herein will provide a foundation for future research efforts aimed at defining and manipulating cellular targets that are critical for the regulation of *TNF* gene expression in the context of i) an innate immune response, and ii) the excessive inflammation found in autoimmune diseases.

Chapter 2

IFN-γ priming of LPS-mediated *TNF* gene expression occurs via inducible IRF1 binding and histone methylation at a highly conserved distal enhancer

2.1 Introduction

During infection, antigen-presenting cells secrete IL-12, which helps link innate and adaptive immunities by promoting the differentiation of naïve $CD4^+$ T cells toward a Th1 phenotype and by inducing IFN-γ secretion from NK cells and activated $CD4^+$ T cells [211]. Accordingly, IFN-γ sensitizes surrounding macrophages to microbial recognition and, most notably, augments the production of TNF [44, 59]. This phenomenon of IFN-γ-mediated macrophage sensitization is known as IFN-γ priming. *In vitro*, maximum effects of IFN-γ priming have been shown to occur within two hours of IFN-γ pre-treatment, resulting in enhanced mRNA levels of TNF [212] and of other cytokines and inflammatory molecules such as IL-12 [60], IL-6 [57], and NO [58] upon LPS stimulation.

The mechanisms involved in priming are unclear; the IFN-γ-inducible transcription factors IRF1 and STAT1 have been implicated [84, 213], but functional roles for these factors have not been determined. One study examining the effect of IFN-γ on chromatin modifica-

tions at the *TNF* promoter observed increased levels of H4ac in primary human monocytes treated with IFN-γ as compared to resting cells [214]. In addition, a recent study has shown H3K27ac enrichment at both the *TNF* promoter and a distal DH site during IFN-γ priming [213]. In the context of IFN-γ priming, functional roles for distal DH sites in the *TNF/LT* locus have not been demonstrated.

Here, we show that IFN-γ priming of *TNF* transcription requires functional binding of IRF1 to the distal *TNF* enhancer element hHS-8. IFN-γ also poises hHS-8 for enhancer function by increasing levels of H3K27me3. Subsequent LPS stimulation triggers hHS-8 enhancer activation by first demethylating and then acetylating H3K27. These experiments provide a mechanistic explanation for IFN-γ priming of the LPS response using TNF as a model system, while at the same time providing potential targets for selective manipulation of TNF overexpression in primed macrophages.

2.2 Results

2.2.1 IFN-γ primes monocytes and macrophages for enhanced *TNF* gene expression.

IFN-γ priming dramatically enhances *TNF* gene expression in LPS-induced monocyte and macrophage activation. We first observed the effect of IFN-γ priming in THP-1 cells, a human monocytic cell line, by pretreating cells with IFN-γ 2h before LPS stimulation and found a two-fold increase in TNF mRNA levels as compared to that of LPS alone (Figure 2.2.1a). To verify that enhanced *TNF* gene expression results in enhanced protein production, we measured TNF protein levels in supernatant and again found a two-fold increase in cells pretreated with IFN-γ as compared to cells treated with LPS alone (Figure 2.2.1b). We also verified that IFN-γ priming is not LPS specific by showing enhanced TNF expression in monocytes activated with whole lysate from *M. tuberculosis* strain H37Rv (Figure 2.2.1c). We next confirmed this phenomenon in primary human MDMs activated by LPS

(Figure 2.2.1d). Despite significant differences in morphology and function between monocytes and macrophages, IFN-γ synergizes with LPS in both cell types to enhance *TNF* gene expression at the transcriptional level, suggesting that IFN-γ affects the chromatin environment at the *TNF/LT* locus in a similar mechanism for both cell types. When proposing a mechanism for IFN-γ priming, we take note that both monocytes and macrophages treated with IFN-γ alone induced minimal levels of both TNF mRNA and protein and were comparable to untreated samples; thus, we hypothesized that although IFN-γ does not induce *TNF* transcription, it poises the *TNF/LT* locus for enhanced transcription in response to LPS stimulation.

2.2.2 IFN-γ promotes chromatin accessibility at hHS-8 in the *TNF/LT* locus.

We next looked at the ability of IFN-γ to remodel the *TNF/LT* locus by performing DNase I hypersensitivity assays (DHAs), and found that IFN-γ promotes DNase I cleavage at the human hypersensitive site, hHS-8. DHAs utilize the ability of DNase I to distinguish and cleave accessible DNA in packaged chromatin. DNA from both THP-1 cells and primary human MDMs stimulated with IFN-γ were partially digested with DNase I. BamHI and ScaI digestion allowed for visualization of the *TNF* promoter and hHS-8, respectively (Figure 2.2.2a). When comparing untreated and IFN-γ treated THP-1 cells, minimal differences in DNase I accessibility at the *TNF* promoter were observed. This is evidenced by similar band intensities between untreated and IFN-γ treated samples (Figure A.0.1a, lane 4 compared to lane 8). However, we detected a strongly enhanced band corresponding to hHS-8 from cells treated with IFN-γ as compared to untreated cells (Figure A.0.1b, lane 4 compared to lane 8). Results were repeated in primary human MDMs for both the *TNF* promoter and hHS-8 (Figures 2.2.2b, lane 3 compared to lane 6; 2.2.2c, lane 3 compared to lane 6). Thus, when thinking of a population of monocytes or macrophages, hHS-8 is constitutively present in a given percentage of cells, and, upon IFN-γ stimulation, the percentage of cells exhibiting

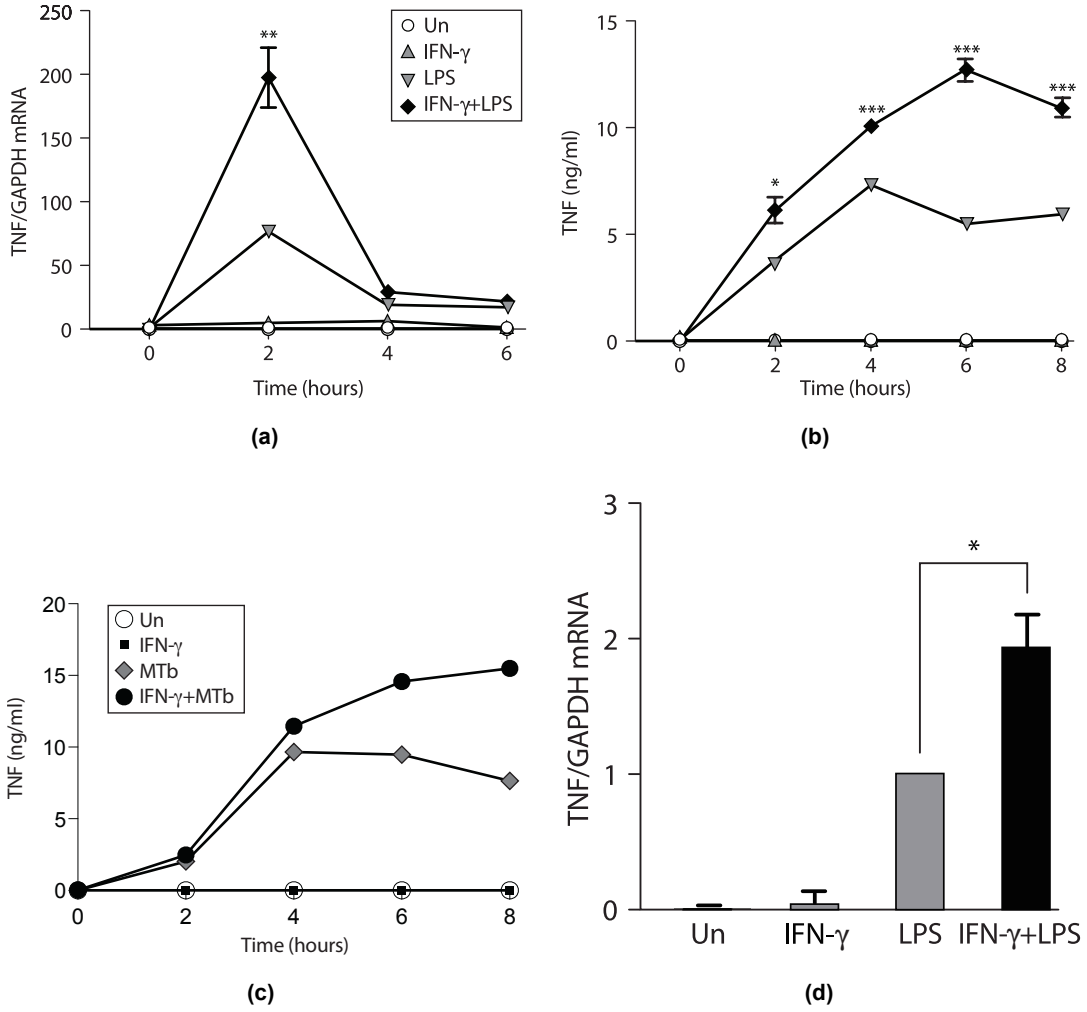


Figure 2.2.1: IFN-γ primes monocytes and macrophages for enhanced *TNF* gene expression. (a) THP-1 cells were stimulated with IFN-γ alone for 3h, LPS alone for 1h, and both IFN-γ and LPS (IFN-γ pretreatment for 2h followed by LPS treatment for 1h), and TNF mRNA levels were measured post LPS stimulation. (b) THP-1 cells were stimulated as in (a), and TNF protein levels in supernatants were measured by ELISA post LPS stimulation. (c) THP-1 cells were stimulated with IFN-γ alone for 3h, MTb lysate alone for 1h, and both IFN-γ and MTb lysate (IFN-γ pretreatment for 2h followed by MTb lysate treatment for 1h), and TNF protein levels in supernatants were measured as in (b). Data from 1 experiment. (d) Primary human MDMs were treated as in (a), and TNF mRNA levels were measured 1h post LPS stimulation. Data from 3 donors. (*) $p \leq 0.05$, (**) $p \leq 0.01$, and (***) $p \leq 0.001$.

accessibility at hHS-8 significantly increases in the population.

In addition to DNase I accessibility, we analyzed nucleosome occupancy at the *TNF* promoter and hHS-8 by measuring total H3 levels by ChIP. As expected, we observed a significant loss in total H3 levels at the promoter in response to LPS stimulation (Figures 2.2.2d, 2.2.2e). We also observed a significant loss in total H3 levels at hHS-8 in response to IFN-γ and LPS stimulation (Figure 2.2.2d). Results were repeated in primary human MDMs isolated from a representative donor (Figure 2.2.2e). This data correlates with our finding that increased DNase I cleavage occurs at hHS-8 in response to IFN-γ treatment. Together, we show that IFN-γ poises the *TNF/LT* locus by exposing hHS-8, a hypersensitive site distal to the *TNF* promoter.

2.2.3 IRF1 binds to the *TNF* promoter and hHS-8 in an IFN-γ-inducible manner.

Increased chromatin accessibility often predicts binding of transcription factors and/or coactivators. We next sought to identify factors binding to the *TNF/LT* locus in an IFN-γ-inducible manner. Given that IFN-γ is a potent inducer of the transcription factor IRF1 [215] (Figure A.0.2), we investigated whether IRF1 binds to specific sequences within the *TNF/LT* locus, particularly at known DH sites. We scanned all DH sites for potential IRF binding sites similar to its consequence sequence (5'-AANNGAAANGAA-3') [82] and verified by both *in vitro* and *in vivo* methods that IRF1 binds to both the *TNF* promoter and hHS-8. Past studies have shown cell type- and inducer-specific recruitment of specific sets of transcription factors and coactivators to the proximal region of the *TNF* promoter (Figure 2.2.3a).

Quantitative DNase I footprinting analysis reveals where factors bind to DNA with the resolution of a single nucleotide. We observed that recombinant IRF1 (rIRF1) binds to the *TNF* promoter in a region ~50bp in length (Figure 2.2.3b). Notably, this is the same region where transcription factors Sp1, Egr1, and NFAT have been shown to bind, which further

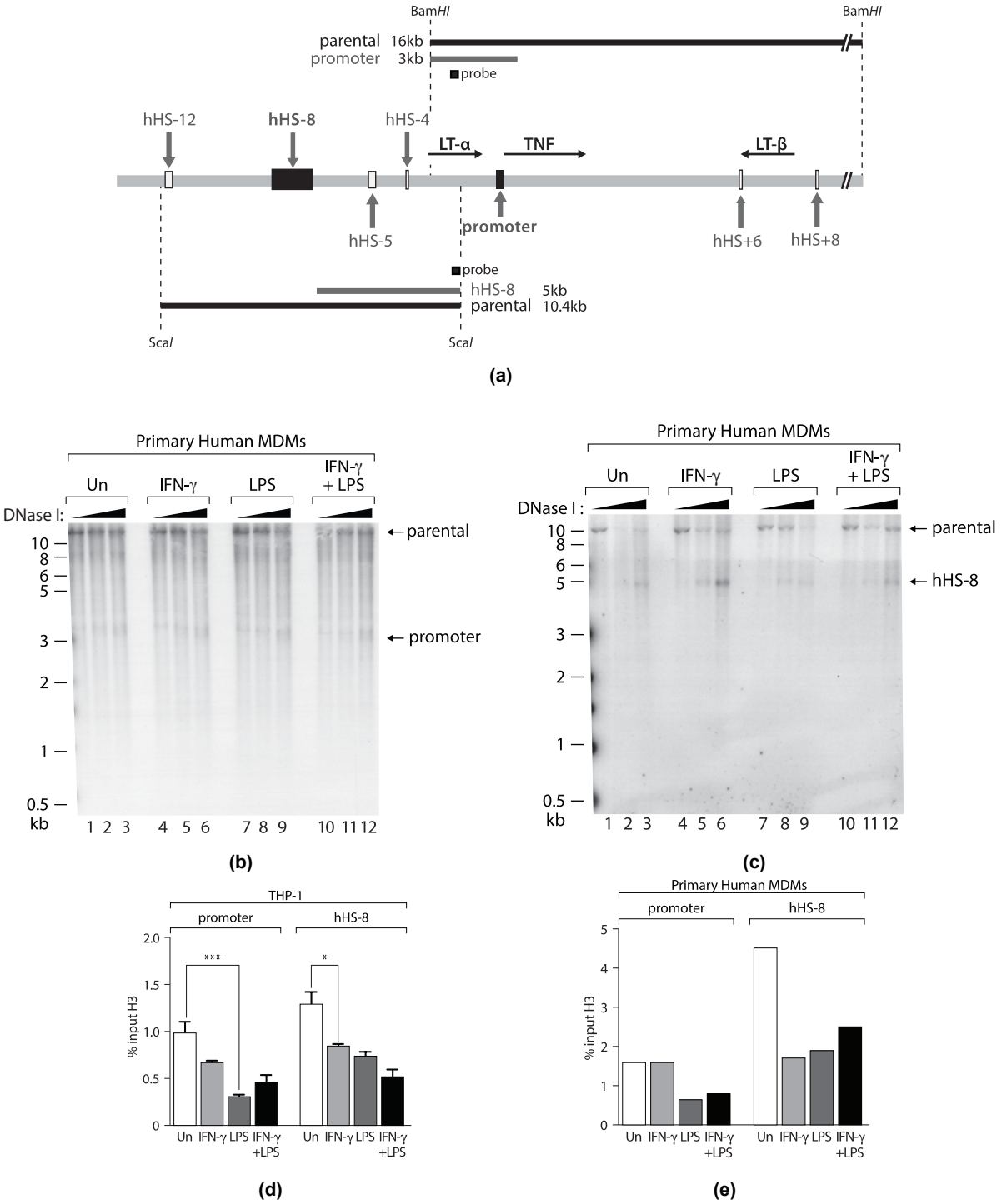


Figure 2.2.2: IFN-γ promotes chromatin accessibility at hHS-8 in the *TNF/LT* locus. (a) Map of the human *TNF/LT* locus. DH sites and positions and directions of transcription of the *TNF*, *LTA*, and *LTB* genes are shown. Positions of the parental ScaI, parental BamHI, and DNase I digestion products for the DHAs are indicated; fragment representing DNase I accessibility at the *TNF* promoter and hHS-8 is 3kb and 5kb in length, respectively. (b) IFN-γ does not affect DNase I cleavage at the *TNF* promoter. DHA using primary human MDMs. (c) IFN-γ strongly increases DNase I cleavage at hHS-8. (d) IFN-γ and LPS decrease nucleosome occupancy at the *TNF* promoter and hHS-8. ChIP using THP-1 cells (d) and primary human MDMs, 1 donor (e) measures total H3 levels. (*) $p \leq 0.05$ and (***) $p \leq 0.001$.

emphasizes the complexity of cell-type and inducer-specific regulation of *TNF* transcription (Figure 1.4.1). In addition to the *TNF* promoter, we observed that rIRF1 occupies a region in hHS-8 that is also ~50bp in length and contains four -GAAA- motifs, suggesting multiple tandem IRF1 binding sites (Figure 2.2.3c). Other *in vitro* assays using human T cells have shown binding of NFAT to regions near the newly identified IRF1 binding site (Figure 2.2.3a).

We next investigated whether IRF1 is recruited to the promoter and hHS-8 in an IFN-γ inducible manner. ChIP studies using primary human MDMs confirmed that upon IFN-γ stimulation, IRF1 is massively recruited to hHS-8 within the same region as identified by footprinting analysis and, to a lesser extent, to the *TNF* promoter (Figure 2.2.3d). Thus, upon IFN-γ stimulation, hHS-8 has a higher affinity for IRF1 than the *TNF* promoter. We speculated that IRF1 recruitment to both the *TNF* promoter and hHS-8 is important for the ability of IFN-γ to prime both monocytes and macrophages.

2.2.4 IRF1 is required for enhanced TNF expression in IFN-γ-primed monocytes and macrophages.

We next tested whether IRF1 plays a functional role in IFN-γ-induced enhancement of *TNF* gene expression. Using bone marrow-derived macrophages (BMDMs) from IRF1-deficient (*Irf1-/-*) mice, we show that IRF1 is required for enhanced *TNF* gene expression in murine macrophages primed with IFN-γ. We isolated BMDMs from wild-type and *Irf1-/-* mice and measured TNF protein levels in response to IFN-γ and LPS. We observed no significant differences in TNF protein levels between wild-type and *Irf1-/-* mice stimulated with IFN-γ and LPS alone. As seen in THP-1 cells and primary human MDMs, wild-type BMDMs secreted significantly higher levels of TNF protein when primed with IFN-γ than when treated with LPS alone (Figure 2.2.4a). However, we did not observe enhancement in *Irf1-/-* BMDMs (Figure 2.2.4b); thus, we conclude that IRF1 is required for IFN-γ-induced enhancement of

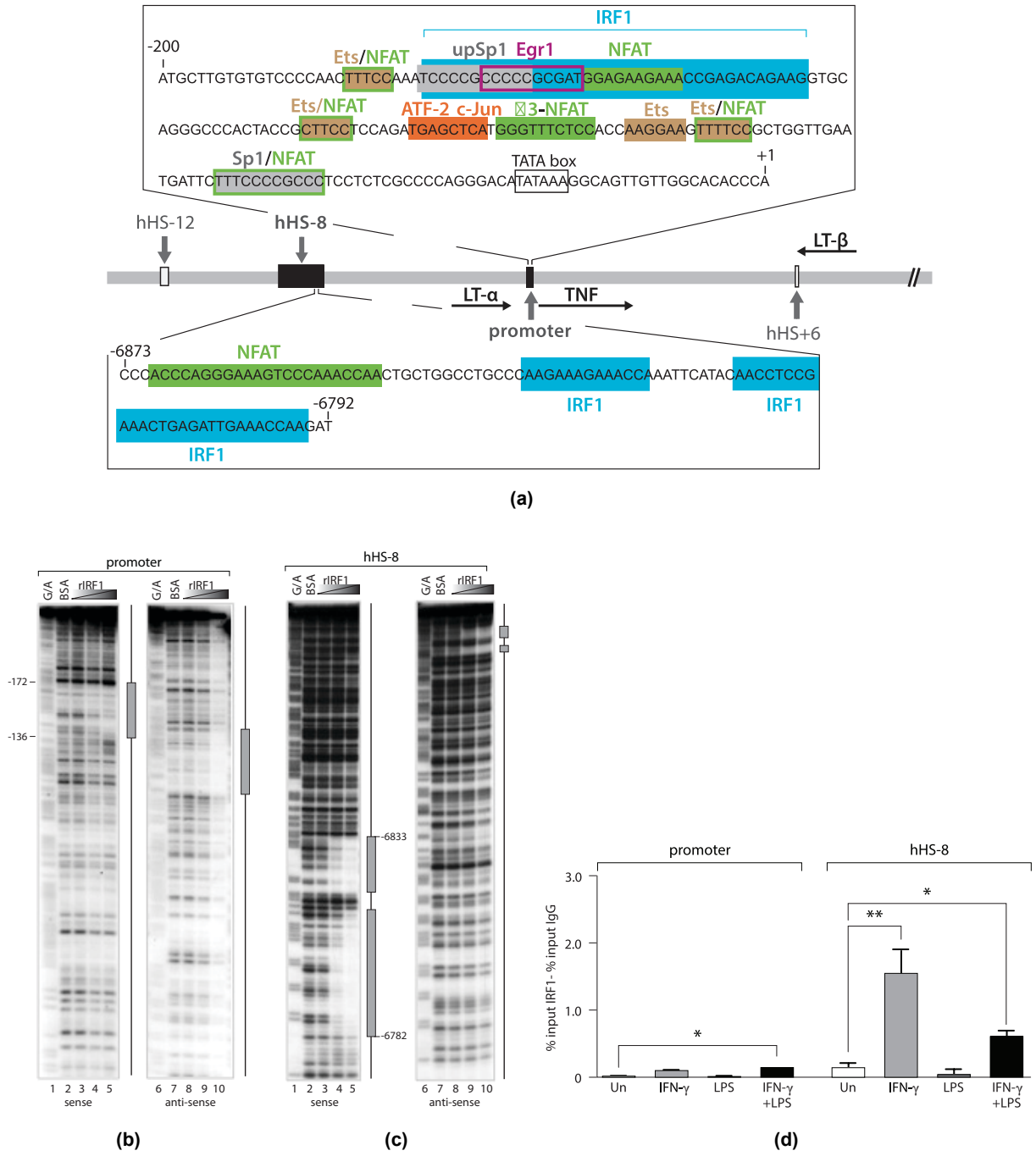


Figure 2.2.3: IRF1 binds to the *TNF* promoter and hHS-8 in an IFN-γ-inducible manner. (a) Human *TNF/LT* locus with partial sequences of both the *TNF* promoter and hHS-8 and positions of transcription factor binding sites. (b) rIRF1 binds to the *TNF* promoter. Quantitative DNase I footprinting analysis of the *TNF* promoter (-200 to +1) and increasing concentrations of rIRF1. Sense and anti-sense strand with G/A ladder and BSA control. Bars mark areas of rIRF1 binding at -172 to -136. (c) rIRF1 binds to hHS-8. Analysis of hHS-8 (-7031 to -6782); bars mark areas of rIRF1 binding at -6833 to -6782. (d) IRF1 is recruited to both the *TNF* promoter and hHS-8 in an IFN-γ-inducible manner. ChIP using primary human MDMs and analyzing IRF1 recruitment, data from 3 separate donors. (*) $p \leq 0.05$ and (**) $p \leq 0.01$.

TNF gene expression.

In addition to murine BMDMs, we looked at the ability of IFN-γ priming to enhance *TNF* gene expression in human monocytes treated with shRNA targeting IRF1. THP-1 cells were transfected with either shRNA targeting human IRF1 or control shRNA encoding a scrambled sequence. Sufficient knockdown of IRF1 mRNA was observed when cells were treated with IFN-γ for 3h (Figure 2.2.4c), which we concluded as sufficient knockdown of IRF1 protein considering its extreme instability (half-life ~30min) [216, 217]. As expected, we observed significant enhancement of TNF mRNA levels in IFN-γ primed THP-1 cells expressing control shRNA. In contrast, enhancement of TNF mRNA levels was lost in primed THP-1 cells expressing IRF1 shRNA (Figure 2.2.4d). These data indicate that IRF1, which we have shown to be massively recruited to hHS-8 upon IFN-γ stimulation, is necessary for enhanced *TNF* gene expression in both murine macrophages and human monocytes primed with IFN-γ. Indeed, the IRF family comprises nine transcription factors that all share similar consensus sequences [82]; however, this data suggests that other IRFs are not functionally redundant to IRF1 in the context of IFN-γ priming.

2.2.5 hHS-8 functions as an IFN-γ-inducible, IRF1-dependent enhancer of *TNF* transcription.

We speculated that hHS-8 might function as an IFN-γ-inducible regulatory element during IFN-γ priming of monocyte and macrophage activation. Specifically, we hypothesized that hHS-8 functions as an IFN-γ-inducible enhancer of *TNF* transcription, and that IRF1 plays a role in the ability of hHS-8 to function as an enhancer. We first analyzed sequence conservation at the IRF1 binding sites within hHS-8 and found these sites to be highly conserved among the great apes, small apes, old world monkeys, and new world monkeys. High sequence conservation within non-coding regions of DNA infers regulatory function [209]. We compared the human sequence to those of non-human primate species and sub-

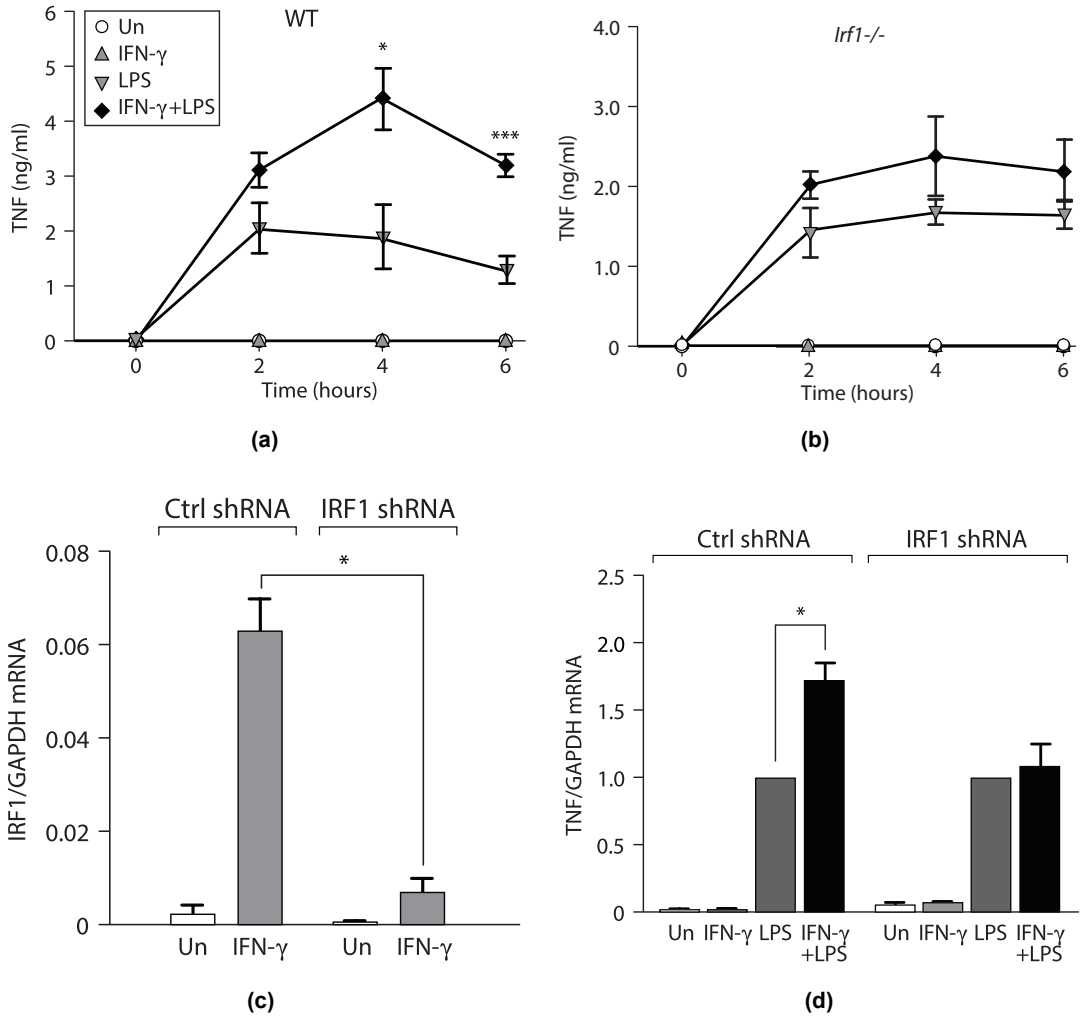


Figure 2.2.4: IRF1 is required for enhanced TNF expression in IFN-γ-primed monocytes and macrophages. (a,b) Enhanced *TNF* gene expression induced by IFN-γ priming is abrogated in IRF1-deficient murine BMDMs. Wild-type (a) and Irf1-/- (b) BMDMs were stimulated, and TNF protein levels in supernatants were measured by ELISA post LPS stimulation. Data from 3 separate experiments each with $N = 3$. (c) THP-1 cells that constitutively express lentivirally-delivered shRNA targeting IRF1 or control shRNA encoding a scrambled sequence were stimulated with IFN-γ alone for 3h, and IRF1 mRNA levels were measured. (d) Enhanced *TNF* gene expression induced by IFN-γ priming is abrogated in human monocytes where IRF1 expression is silenced. THP-1 cells expressing IRF1 and control shRNA were stimulated, and TNF mRNA levels were measured (shown relative to LPS values). (*) $p \leq 0.05$ and (***) $p \leq 0.001$.

species including multiple individuals representing species of the great apes [chimpanzee (*Pan troglodytes*), gorilla (*Gorilla*), and orangutan (*Pongo*)], the small apes [two genera of gibbon (*Hylobatidae* and *Nomascus*)], the old world monkeys [macaque (*Macaca*), *Chlorocebus*, and *Papio*] and the new world monkeys [*Callithrix* and *Lagothrix*]. We found that the core -GAAA- motifs of the IRF1 binding sites in hHS-8 are completely conserved among human and all non-human primate species analyzed (Figure 2.2.5a), which suggests the presence of phylogenetic footprints in hHS-8. High sequence conservation among primates is similarly seen in the *TNF* promoter where multiple binding sites for transcription factors and coactivators are involved in highly coordinated cell type- and inducer-specific enhanceosome formation [197, 210]. Notably, analysis of IRF1 binding sites in HSS-9 (murine hHS-8) showed two differences in the core -GAAA- motifs (Figure 2.2.5a). Through EMSA analysis, we demonstrated that the differences in the mouse sequence do not impact IRF1 binding (Figure A.0.3, lane 2 compared to lane 4).

In order to test whether hHS-8 functions as an inducible enhancer of *TNF* transcription, we cloned the hHS-8 sequence (Figure A.0.4) into a reporter vector where luciferase expression is driven by the *TNF* promoter. We transiently co-transfected the constructs with pRL-TK (Renilla luciferase, internal control) into the murine macrophage cell line J774 and stimulated cells accordingly. Luciferase activity was measured by a dual-luciferase reporter assay. In macrophages primed with IFN-γ, the presence of hHS-8 significantly enhanced the ability of the *TNF* promoter to drive luciferase expression in response to LPS, confirming that hHS-8 functions as an IFN-γ-inducible enhancer.

In order to assess the importance of IRF1 in hHS-8 enhancer function, we also mutated all four highly conserved IRF1 -GAAA- motifs to -GAGG-, which we showed by EMSA analysis to completely disrupt IRF1 binding (Figure 2.2.5b). Mutation of the IRF1 binding sites in hHS-8 abolished its ability to enhance *TNF* promoter activity in macrophages primed by IFN-γ and induced by LPS (Figure A.0.5); thus, we conclude that hHS-8 functions as an

IRF1-dependent inducible enhancer of *TNF* transcription in macrophages primed by IFN-γ. Understanding the functional importance of IRF1 binding to the *TNF* promoter is complicated by the fact that LPS induced *TNF* transcription requires intact Sp1 and Egr binding sites [189] that were found to coincide with the IRF1 binding sites (Figure 2.2.3a). Thus, we only examined the functional importance of IRF1 binding to hHS-8.

2.2.6 H3K27me3 and H3K27ac play a critical role in IFN-γ priming.

H3K27me3 and H3K27ac have been shown to distinguish active and inactive enhancers in mouse and human embryonic stem cells [218–220]. We hypothesized that IFN-γ priming and LPS stimulation affect H3K27me3 and H3K27ac levels at hHS-8. ChIP studies using THP-1 cells showed that upon IFN-γ stimulation, levels of H3K27me3 increased at hHS-8, whereas levels at the *TNF* promoter remained constant (Figure 2.2.6a). Cells that were primed with IFN-γ and then stimulated with LPS were not enriched for H3K27me3 at hHS-8, suggesting that H3K27me3 marks hHS-8 during priming and is lost upon *TNF* gene activation. Enrichment of H3K27ac was also shown to be inducible; levels of H3K27ac significantly increased at hHS-8 in cells primed by IFN-γ and stimulated with LPS (Figure 2.2.6b). Notably, H3K27ac enrichment at hHS-8 did not occur in cells treated with LPS alone, demonstrating a requirement of IFN-γ priming for H3K27ac enrichment at hHS-8. H3K27ac levels at the *TNF* promoter dramatically increased upon stimulation with LPS alone, which supports the finding of LPS-induced CBP/p300 recruitment to the *TNF* promoter in monocytes and macrophages [189].

Next, we investigated the reciprocal nature of H3K27me3 and H3K27ac at hHS-8 and whether the marks were important for enhanced *TNF* gene expression in the context of IFN-γ priming. To do this, we inhibited the H3K27me3-specific demethylases JMJD3 and UTX. THP-1 cells were first primed by IFN-γ and then treated with either GSK-J4 [147] (active inhibitor of JMJD3 and UTX) or GSK-J5 [147] (inactive inhibitor) 30min before the LPS

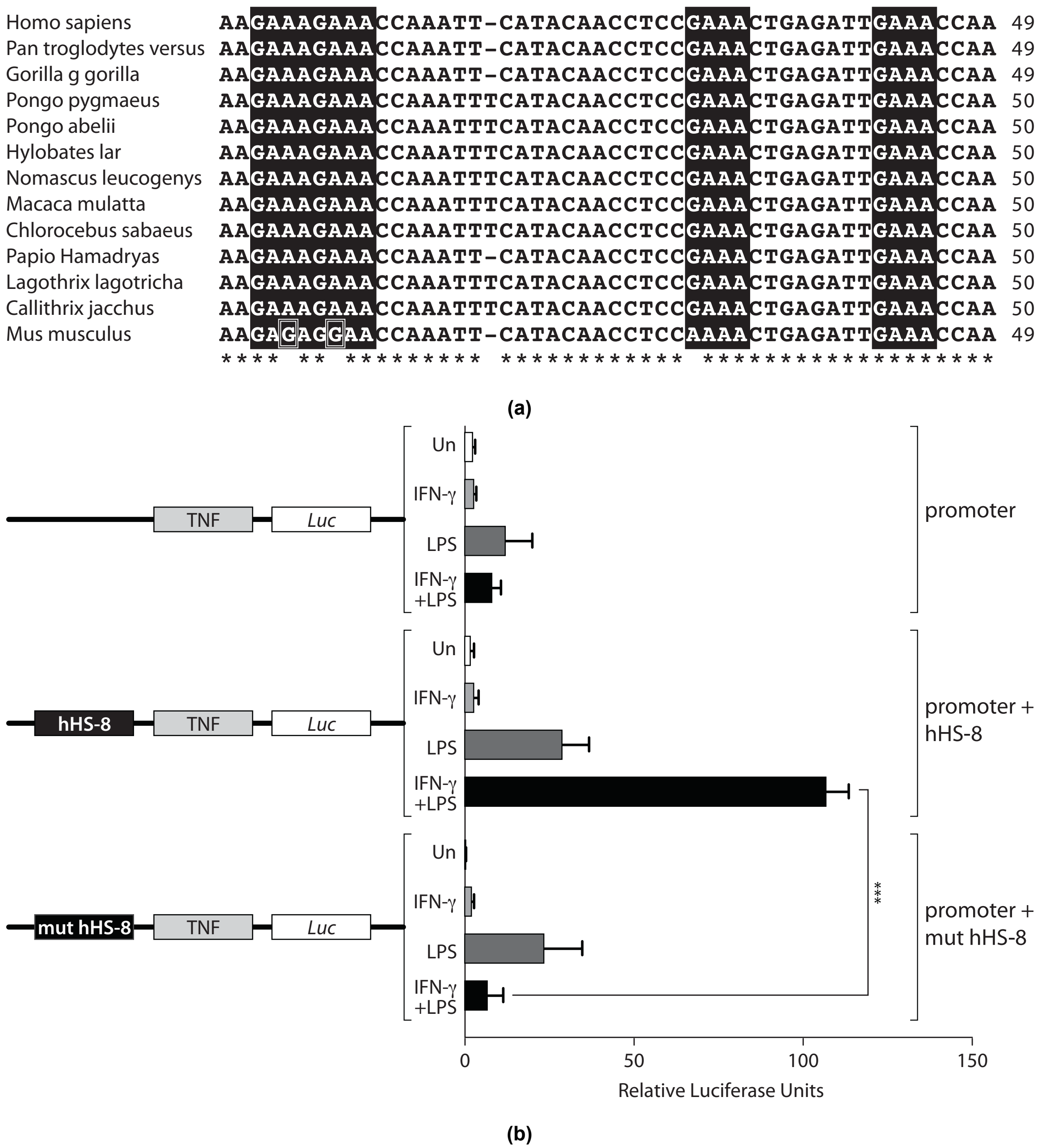


Figure 2.2.5: hHS-8 functions as an IFN-γ-inducible, IRF1-dependent enhancer of *TNF* transcription. (a) IRF1 binding sites (nucleotides -6833 to -6784) in hHS-8 are highly conserved among all primate species examined. Critical -GAAA- motifs for IRF1 binding are highlighted. (b) Disruption of IRF1 binding to hHS-8 abolishes inducible enhancer function and thus enhanced *TNF* transcription induced by IFN-γ priming. Constructs were transfected into J774 cells and stimulated with IFN-γ alone for 8h, LPS alone for 6h, and both IFN-γ and LPS (IFN-γ pretreatment for 2h followed by LPS treatment for 6h). “TNF” is the *TNF* promoter and “hHS-8” is the entire sequence of hHS-8 (1250bp in length), and “muthHS-8” is hHS-8 with mutations that disrupt IRF1 binding. (***) $p \leq 0.001$.

stimulations (Figure 2.2.6c). Notably, THP-1 cells are deficient in UTX expression [221] and thus serve as an effective model system for understanding the effects of specifically inhibiting JMJD3 with the compound. In cells treated with GSK-J5, we observed effective IFN-γ priming, evidenced by enhanced TNF mRNA levels upon LPS induction (Figure 2.2.6d). In contrast, IFN-γ priming failed to enhance TNF mRNA levels in cells treated with GSK-J4, demonstrating a critical role for H3K27me3 in IFN-γ priming and the ability to enhance *TNF* transcription. We hypothesize that IFN-γ poises hHS-8 for enhancer function through mechanisms that involve IRF1 recruitment and H3K27me3 enrichment. Subsequent LPS stimulation activates hHS-8 enhancer function through mechanisms that first demethylate and then acetylate H3K27.

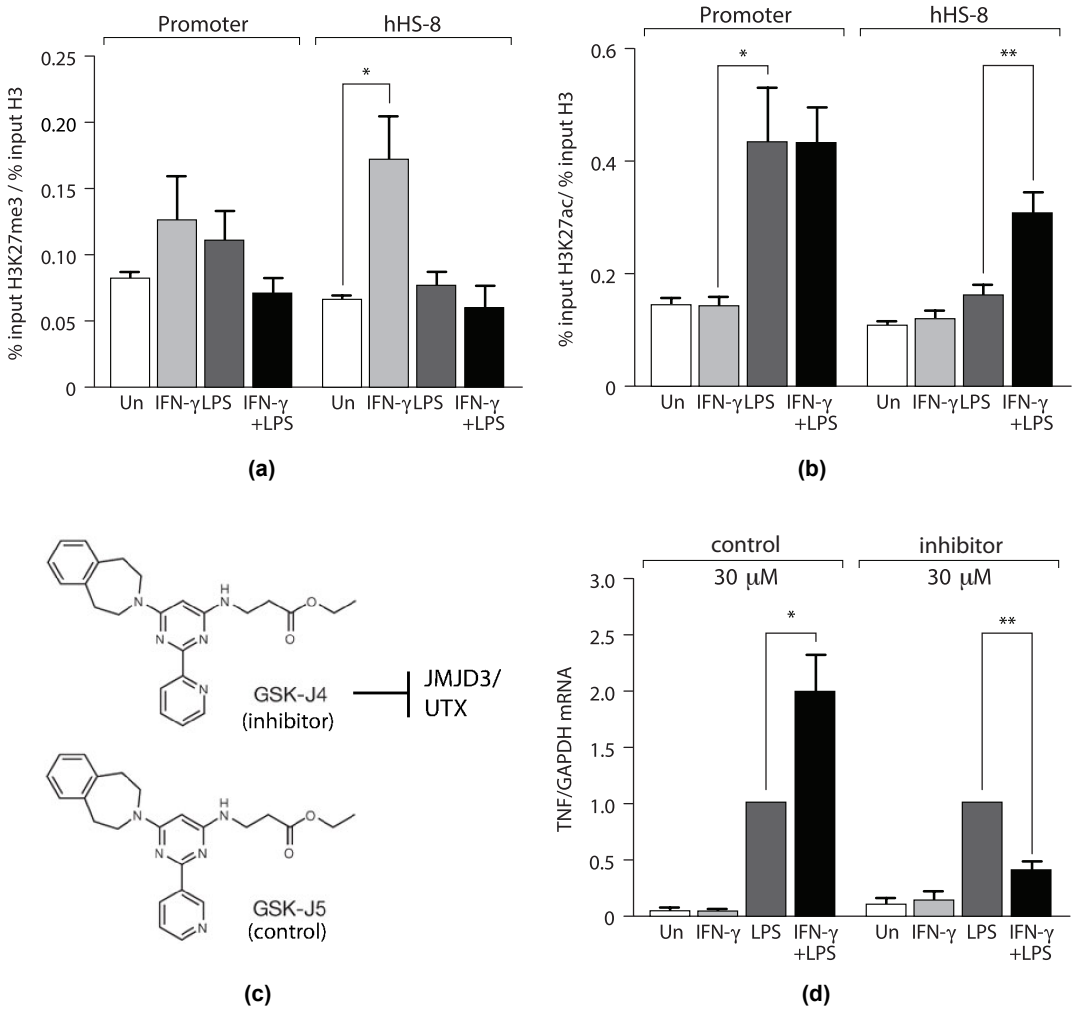


Figure 2.2.6: H3K27me3 and H3K27ac play a critical role in IFN-γ priming. (a) IFN-γ increases H3K27me3 prevalence at hHS-8. ChIP using THP-1 cells and analyzing H3K27me3 prevalence at the *TNF* promoter and hHS-8. (b) Activation of hHS-8 enhancer function corresponds with increased H3K27ac prevalence at hHS-8. ChIP using THP-1 cells and analyzing H2K37ac prevalence at the *TNF* promoter and hHS-8. (c) Chemical structures of the active JmjD3/UTX inhibitor (GSK-J4) and the inactive inhibitor (GSK-J5). (d) Inhibition of H3K27me3 demethylation prevents enhanced *TNF* gene expression induced by IFN-γ priming. THP-1 cells were treated with either the GSK-J5 or GSK-J4 compound (30uM) 30min before LPS stimulations, and TNF mRNA levels were measured (shown relative to LPS values). (*) $p \leq 0.05$ and (**) $p \leq 0.01$.

2.3 Discussion

One of the most important functions of IFN-γ is to prime monocytes and macrophages thereby ensuring an enhanced response to microbial activation. The mechanism of IFN-γ priming has been investigated for IL-12 [84] and IL-6 [57], and in both cases, transcriptional regulation has been confined to the promoter. Considering the importance of TNF and its dysregulation in the pathology of several inflammatory disorders, we sought to determine the mechanism for IFN-γ-induced priming of *TNF* gene expression. Here, we observe transcriptional regulation by IFN-γ to be beyond the promoter by demonstrating a critical role for an inducible enhancer distal to the *TNF* TSS. In addition to showing a prominent role for IRF1 in enhancer function, we observe H3K27me3 marking a poised enhancer in an adult somatic cell in the context of immune signaling.

Spanning ~1.3kb, hHS-8 is nearly five times larger than the average non-promoter DH site [122], and we have shown functional IRF1 recruitment to its most highly conserved region. We do expect hHS-8 enhancer function to additionally involve the recruitment of coactivator proteins and multiple lineage-specific transcription factors. Indeed, studies in murine T cells have shown NFAT binding to HSS-9 [177]. Considering the necessity of IRF1 for enhanced *TNF* gene expression in primed monocytes and macrophages, we wonder whether IRF1 functions as a pioneer factor. First identified in yeast, pioneer factors are transcription factors that bind to DNA and recruit chromatin-remodeling complexes thereby promoting the recruitment of other factors to their corresponding DNA sequences that otherwise would have been inaccessible [165]. Additional binding studies in monocytes and macrophages are required in order to properly assess whether IRF1 functions as a pioneer factor. Indeed, IRF1 has been reported to interact with the histone acetyltransferase, CBP/p300 [222]. In addition, we have shown by ChIP analysis that p300 is recruited to hHS-8 upon LPS stimulation in primary human MDMs (Figure A.0.6). We can imagine

a situation where IFN-γ utilizes IRF1 to recruit CBP/p300 to hHS-8; CBP/p300, along with the aid of other factors, could induce chromatin-remodeling events necessary for enhancer activation.

Enhancers are often characterized by chromatin signatures such as increased chromatin accessibility, enrichment of specific histone modifications like H3K4me1 and H3K27ac, prevalence of RNA Pol II, presence of histone variants H3.3 and H2A.Z, and increased prevalence of ATP-dependent chromatin remodeling complexes like CBP/p300 [223]. Recent studies in embryonic stem cells have shown that only a fraction of elements marked by H3K4me1 are actively engaged in enhancing transcription; thus, H3K4me1 does not distinguish between active and inactive/poised enhancers. The presence of H3K27ac has been extensively shown in both human and mouse embryonic stem cells to separate active from poised enhancers [218]. Specifically, active enhancers are enriched for H3K27ac while poised enhancers lack H3K27ac enrichment. In addition to lacking H3K27ac, poised enhancers have been shown, most often, in pluripotent cells to be enriched for H3K27me3 [219, 220]. Here, we show that in an adult somatic cell, IFN-γ marks a poised enhancer in the *TNF/LT* locus with H3K27me3 thereby supporting the finding that H3K37me3 enrichment at poised enhancers is not limited to pluripotent cells. We also show that upon LPS stimulation, levels of H3K27me3 are significantly decreased at hHS-8; this is followed by an enrichment of H3K27ac, which suggests enhancer activation.

What then is the mechanism for LPS-induced removal of H3K27me3 and addition of H3K27ac at hHS-8? We hypothesize that the same factor(s) responsible for demethylating H3K27 form a complex with the factor(s) responsible for acetylating H3K27. Interestingly, a study in *Drosophila* showed that UTX physically associated with CBP *in vivo* [224], suggesting that the individual activities of demethylating and acetylating H3K27 are functionally coupled. We hypothesize that upon LPS stimulation, complexes containing JMJD3 (or UTX) and CBP/p300 associate with hHS-8 and effectively demethylate and acetylate

H3K27 residues. This in turn is essential for hHS-8 enhancer activation.

In addition, investigating whether IRF1 recruitment to hHS-8 plays a functional role in the enrichment of H3K27me3 and H3K27ac may provide novel insights regarding the role of transcription factors in enhancer activation. ChIP analysis of H3K27me3 and H3K27ac in primed monocytes and macrophages where IRF1 expression is knocked down could provide insight into the many functions of IRF1. We would like to note that GSK-J4 targets global H3K27me3 demethylation and that it is difficult to understand the effects of this modification at one particular region of DNA. Thus experiments targeting the disruption of H3K27me3 demethylation specifically at hHS-8 would need to be employed.

In our model, we predict the formation of an IRF1-dependent intrachromosomal interaction at the *TNF/LT* locus between the promoter and hHS-8 upon TLR-induced activation in monoctyes and macrophages primed by IFN-γ. The mechanism(s) of action on promoters for enhancers has yet to be determined. The predominant model known as the "looping" model predicts a direct interaction between the promoter and enhancer resulting in the displacement or "looping out" of the intervening DNA [169]. Using chromosome conformation capture (3C) assay, an inducible intrachromosomal interaction between HSS-9, HSS+3, and the *TNF* promoter was demonstrated in murine T cells [177]. Unfortunately, we were unable to successfully perform the 3C assay using our model system. Other, less robust techniques suggesting a direct interaction between a promoter and enhancer involve Pol II occupancy at the enhancer. We have yet to investigate whether Pol II is recruited to hHS-8 upon TLR-activation in monoctyes or macrophages primed by IFN-γ. Other potential mechanisms of enhancer action include enhancer RNAs (eRNAs) [225]. eRNAs are transcripts that are products of transcription at Pol II-associated enhancers; they can either by polyadenylated or non-polyadenylated. The fact that not all enhancers are transcribed implies functional differences between transcribed and non-transcribed enhancers. It is thought that transcription of enhancers, similar to H3K27ac enrichment, might mark

active enhancers; however, the functional roles, if any, for eRNAs are unknown. It is of interest to investigate whether hHS-8 is transcribed.

In conclusion, our identification of critical protein-DNA interactions and inducible histone modifications during IFN-γ priming of LPS-induced *TNF* transcription not only provides a mechanism of IFN-γ priming but also provides potential therapeutic targets to control TNF dysregulation. Future studies will characterize additional factors and interactions that regulate this vital immune process.

2.4 Materials and Methods

Cell culture and stimulations

THP-1 cells were maintained in RPMI-1640 supplemented with 10% FBS. J774 cells were maintained in DMEM supplemented with 10% FBS. For primary human MDMs, enriched populations of human monocytes were isolated from healthy human donor buffy coats using a $CD14^+$ positive selection kit (Stemcell). MDMs were obtained after 6d of culture in RPMI-1640 medium supplemented with 5% human serum AB (Gemcell) and GM-CSF (50ng/ml; Peprotech). Cells were treated with IFN-γ (100ng/ml; R&D) and LPS (100ng/ml; Sigma E. coli O111:B4). GSK-J4 and GSK-J5 compounds [147] were generously supplied by GlaxoSmithKline, resuspended in DMSO, and used at a final concentration of 30μM.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from cells with a QuickRNA Mini kit (Zymo) and treated with Turbo DNA-free kit (Invitrogen). cDNA was synthesized from total RNA with M-MLV reverse transcriptase (Invitrogen) and 20-residue oligo (dT) (Invitrogen). TNF mRNA levels were measured by the change-in-threshold ($\Delta\Delta$Ct) method based on quantitative real-time PCR in an iCycler iQ (Bio-rad) with SyberGreen Master Mix (Invitrogen) and primers rec-

Table 2.4.1: Primer sequences.

Primer	*Sequence*
Human TNF exon 4 (F)	TCTTCTCGAACCCCGAGTGA
Human TNF exon 3 (R)	CCTCTGATGGCACCACCAG
Human GapdH (F)	GGTGGTCTCCTCTGACTTCAACA
Human GapdH (R)	GTTGCTGTAGCCAAATTCGTTGT
Human IRF1 (F)	GCCAGTCGACGAGGATGAGGAAGGG
Human IRF1 (R)	CCAGCGGCCGCCTGCTACGGTGCAC
ChIP IRF1, -244 to -82 (F)	GGGAGTGTGAGGGGTATCCT
ChIP IRF1, -244 to -82 (R)	CATTCAACCAGCGGAAAACT
ChIP IRF1, -6842 to -6737 (F)	GGCCTGCCCAAGAAAGAAACCAAA
ChIP IRF1, -6842 to -6737 (R)	ACGTGCATGTGAGATATGCGAAGG
ChIP TaqMan, *TNF* promoter (F)	GCGATGGAGAAGAAACCGAGACAGAA
ChIP TaqMan, *TNF* promoter (R)	GGGTGTGCCAACAACTGCCTTTAT
ChIP TaqMan, *TNF* promoter (probe)	/56-FAM/AGCTCATGG/ZEN/GTTTCTCCACCAAGGAA/3IABkFQ/
ChIP TaqMan, hHS-8 (F)	GGCCTGCCCAAGAAAGAAACCAAA
ChIP TaqMan, hHS-8 (R)	ACGTGCATGTGAGATATGCGAAGG
ChIP TaqMan, hHS-8 (probe)	/56-FAM/AACCAAGAT/ZEN/TGGCCCATCTCAAGGAGCA/3IABkFQ/

ognizing exon 4 and exon 3 of the human *TNF* gene, the human *GapdH* gene, and the human *IRF1* gene (Table 2.4.1).

Measurement of TNF production

TNF secretion was quantified by ELISA specific for murine and human TNF (R&D) using a VMax®Kinetic ELISA Microplate Reader.

DNase I Hypersensitivity Assay (DHA)

DHAs were performed using both THP-1 cells and primary human MDMs. MDMs were detached from culture surface using TrypLE™ (Life Technologies). Cells were harvested, washed with PBS, and resuspended in RSB buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, and 3mM MgCl2). Cells were lysed with lysis buffer (0.5% NP-40 in 1x RSB buffer) on ice for 5min. Resuspended nuclei in RSB buffer were treated with DNase I (40ng/ul) at

37C for 5min. DNase I activity was quenched upon addition of stop solution (0.6M NaCl, 20mM Tris-HCl pH 8.0, 10mM EDTA, and 1% SDS). Samples were treated with Proteinase K at 56C O/N. DNA was digested with ScaI and BamHI restriction enzymes and analyzed by Southern blotting using a radiolabeled P^{32} probe corresponding to the coding region of LT-α. 10ug of DNA was used for each lane.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with anti-IRF1 (H-205, Santa Cruz), Rb IgG (Diagenode), anti-H3K27me3 (C36B11, Cell Signaling), anti-H3K27ac (Active Motif), and anti-H3 (Abcam). THP-1 cells and primary human MDMs were treated (IFN-γ 100ng/ml, LPS 100ng/ml), fixed with 10% formaldehyde for 15min, treated with 2.5M glycine for 5min, harvested, washed with PBS, lysed with 0.25% Triton X-100 and 0.5% NP-40 for 5min, centrifuged at 1200RPM for 10min, resuspended in 1% SDS lysis buffer, and sonicated 5min for 4 cycles in a Biorupter. Sonicated DNA was set up for immunoprecipitation O/N and DNA-protein complexes were recovered by adding Protein A/G Plus Agarose Beads (Thermo Scientific) for 3h. Samples were washed 6 times with 1ml of wash buffer and treated with proteinase K at 65C O/N. Samples were treated with phenol/chloroform before O/N ethanol precipitation. DNA fragments for IRF1 recruitment were analyzed by quantitative real-time PCR with Sybergreen master mix (Invitrogen) and primer sets for regions -244 to -82 (promoter) relative to the *TNF* TSS and -6842 to -6737 (hHS-8) relative to the *TNF* TSS (Table 2.4.1). Rb IgG percent input values were subtracted from IRF1 percent input values. DNA fragments for H3K27me3 and H3K27ac analysis were analyzed by quantitative real-time PCR using Jumpstart™ Taq ReadyMix™ For Quantitative PCR (Sigma) and primer/probe sets for the *TNF* promoter and hHS-8 (Table 2.4.1). H3K27me3 and H3K27ac percent input values were normalized to H3 percent input values.

DNase I Footprinting Assay

Radiolabeled P^{32} fragments of the *TNF* promoter (-200 to +1) and hHS-8 (-7031 to -6782) regions were incubated with recombinant IRF1 protein (Abcam) and treated with diluted DNase I at RT for 5s before quenching enzyme activity with stop solution (0.13mM EDTA, 0.5% SDS, and tRNA). Samples were treated with phenol chloroform and DNA was precipitated O/N at -20C. G/A ladder was treated with 4% formic acid, radiolabeled with P^{32}, treated with 1M piperdine, and precipitated with n-butanol. DNA fragments were separated by electrophoresis on an 8% sequencing gel.

Mice

6-8 week old C57BL/6J mice and B6.129S2-Irf1^{tm1Mak}/J mice were purchased from Jackson Laboratories. Experimental procedures were done in accordance with the Institutional Animal Care and Use Committee (IACUC) and the Harvard Medical Area Standing Committee on Animals (HMA IACUC).

Isolation, culture, and stimulation of murine BMDMs

For the generation of murine bone marrow-derived macrophages (BMDMs), bone marrow cells of wild type or *Irf1-/-* mice (6-8wks) were cultured in DMEM medium supplemented with 10% FBS, 10% L929 cell conditioned medium (LCCM), 100U/ml penicillin, 100ug/ml streptomycin, and 2mM L-glutamine. Cells were fed on day 5 and media was changed on day 7, 3h before mIFN-γ (100ng/ml; R&D) and LPS (100ng/ml; Sigma E. coli O111:B4) treatment. Supernatant was collected 2, 4, and 6h after treatment.

Short hairpin RNA

The lentiviral plasmid PLKO.1 expressing shRNA targeting human IRF1 was purchased from the RNAi Consortium (TRC) Lentiviral shRNA library (Thermo Scientific). Clone

TRCN0000014668 with a target sequence of CGTGTGGATCTTGCCACATTT was validated in our laboratory. Control shRNA encodes a scrambled sequence. Lentiviruses encoding shRNA sequences were generated by transfecting the packaging cell line HEK-293T with the shRNA-encoding pLOK.1 plasmids in combination with the packaging plasmid psPAX2 and the envelope plasmid pMD2.G using Effectene transfection reagent (Qiagen). Supernatants were collected 48h post-transfection, clarified by centrifugation, and stored at -80C. THP-1 cells were transduced with the lentiviral particles by culturing the cells with supernatants from the virus-producing cells in the presence of 8ug/ml polybrene (Millipore) and spinoculation for 2h at 2000RPM. Successfully transduced cells were selected and expanded by treatment with 0.8ug/ml puromycin.

Electrophoretic Mobility Shift Assay (EMSA)

Radiolabeled P^{32} oligonucleotides were added to THP-1 nuclear extracts or recombinant IRF1 protein (Abcam) in a binding buffer solution (10mM Tris-HCl pH 7.5, 53mM NaCl, 1mM DTT, 0.01% Nonidet-P40, 5% glycerol, and 0.05ug/ul of double-stranded poly(dI-dC)) at RT for 30min. In super-shift experiments, samples were incubated with 2ug of anti-IRF1 (H-205, Santa Cruz Biotechnology). Protein-DNA complexes were separated by electrophoresis on a 5% PAAG gel.

Sequencing

Procurement of cell lines and samples of blood or DNA from representative individuals of the primate species and subspecies was previously described [197, 210]. Genomic DNA was isolated using QIAamp DNA Blood kit (Qiagen). Sequence alignments were performed using ClustalW2 multiple sequence alignment provided by EMBL-EBI.

Plasmids

Construction of the *TNF* promoter-driven luciferase reporter was previously described [189]. The *TNF* promoter with hHS-8 plasmid was constructed by inserting nucleotides -7833 to -6583 relative to the *TNF* TSS into the *TNF* promoter-driven luciferase reporter construct using MluI and NheI restriction enzyme sites. The *TNF* promoter with mutated hHS-8 plasmid was constructed by circular site-directed mutagenesis.

Luciferase Reporter Assay

J774 cells were transfected with luciferase reporter constructs using an Effectene Transfection Reagent kit (Qiagen). Cells were treated with mIFN-γ (100ng/ml; R&D) and LPS (100ng/ml, Sigma E. coli O111:B4). Luciferase assays were performed 8h after treatment under the Dual Luciferase Reporter Assay System (Promega) using a Dynex luminometer and Renilla luciferase (pRL-TK) as a control.

Chapter 3

Characterization of chromatin alterations at the *TNF/LT* locus during endotoxin tolerance and its IFN-γ-mediated abrogation

3.1 Introduction

Sepsis is a major cause of death in modern intensive care units (ICUs) and accounts for a huge financial burden on public health care systems [226]. It is caused by severe infection and is classically characterized by an initial phase of uncontrolled systemic inflammation that is uniquely followed by a prolonged immunosuppressive state [227–230]. Indeed, this later, immunosuppressive phase frequently correlates with high risks of secondary infection and mortality during the patient's ICU stay [231]. Studies have demonstrated that blood monocytes isolated from sepsis patients who have entered the immunosuppressive phase share several characteristics with endotoxin-tolerant monocytes and macrophages [232–235]. Thus, an understanding of how monocytes and macrophages "switch" from inflammatory to immunosuppressive states could help clinicians more efficiently target the later, more severe stages of sepsis.

As stated previously, endotoxin tolerance is the phenomenon in which immune cells, primarily monocytes and macrophages, transiently become hyporesponsive or "tolerant" upon

repeated or prolonged exposure of LPS [64]. In the case of sepsis, monocytes isolated from sepsis patients and subsequently stimulated with LPS were shown to express significantly lower levels of inflammatory cytokines like Il-1β, IL-6, and especially TNF, as compared to LPS-induced monocytes from healthy individuals [232].

One study examining epigenetic modifications at individual promoters in murine macrophages demonstrated that chromatin modifications distinguish two distinct classes of genes during endotoxin tolerance."Class T" comprised genes that were tolerant upon repeated challenges of LPS while "class NT" comprised genes that remained inducible upon repeated challenges of LPS [236]. Importantly, "class T" promoters in contrast to "class NT" promoters remained primarily deacetylated and failed to recruit the chromatin remodeling protein Brg1. This and the work of others demonstrate the importance of examining chromatin modifications at individual promoters when determining mechanisms for selective modulation of inflammatory responses [237, 238].

Notably, IFN-γ priming has been shown to abrogate endotoxin tolerance, thereby restoring responses to LPS stimulation in monocytes and macrophages [73]. Using primary human monocytes, IFN-γ priming was shown to restore *IL6* transcription by promoting chromatin accessibility at the human *IL6* promoter [239]. Thus, the mechanisms responsible for the induction and abrogation of endotoxin tolerance involve chromatin remodeling. Few studies have focused on *TNF* gene regulation in the context of endotoxin tolerance [239–241] and none to our knowledge have looked beyond the promoter. Here, by examining the *TNF/LT* locus, we argue that alterations in chromatin modifications at the *TNF* promoter and distal DNA elements play a major role in the induction and IFN-γ-mediated abrogation of endotoxin tolerance. Specifically, we have shown that H3K27me3 marks the minimal *TNF* promoter in endotoxin-tolerant macrophages. In addition, we look beyond the *TNF* promoter and observe the appearance of an IFN-γ-inducible DH site that is ~4kb upstream of the *TNF* TSS.

3.2 Results

3.2.1 IFN-γ restores *TNF* gene expression in endotoxin-tolerant monocytes and macrophages.

To induce endotoxin tolerance, we treated primary human MDMs with LPS (100ng/ml) for 24h followed by a second challenge of LPS (100ng/ml) (Figure A.1.1). From past *in vitro* studies, methods for inducing endotoxin tolerance vary with regards to the number of LPS challenges and the concentration of LPS used for each challenge. Despite these variations in experimental design, it is generally accepted that repeated or prolonged exposure of LPS induces endotoxin tolerance in monocytes and macrophages. We next measured TNF mRNA levels from both endotoxin-tolerant (T) and non-tolerant (LPS) MDMs stimulated with LPS. We observed a significant decrease in mRNA levels from endotoxin-tolerant MDMs as compared to non-tolerant MDMs, which suggests that the mechanism(s) of endotoxin tolerance targets *TNF* transcription (Figure 3.2.1a). We also observed the effects of IFN-γ priming by pretreating endotoxin-tolerant MDMs with IFN-γ 2h before the second challenge of LPS (Figure A.1.1). As reported in primary human monocytes [239], IFN-γ priming partially restores the TNF response in endotoxin-tolerant MDMs (Figure 3.2.1a). We also confirmed the induction and abrogation of endotoxin tolerance in THP-1 cells, a human monocytic cell line, using the same experimental design (Figure 3.2.1b).

3.2.2 IFN-γ abrogates endotoxin tolerance in an IRF1-independent manner.

Considering the mechanism that we have defined for IFN-γ priming in non-tolerant THP-1 cells and primary human MDMs, we sought to understand whether IRF1 plays a major role in the ability of IFN-γ to restore *TNF* gene expression in endotoxin-tolerant cells. THP-1 cells were transfected with either shRNA targeting human IRF1 or control shRNA encoding a scrambled sequence. Sufficient knockdown of IRF1 mRNA was observed when cells

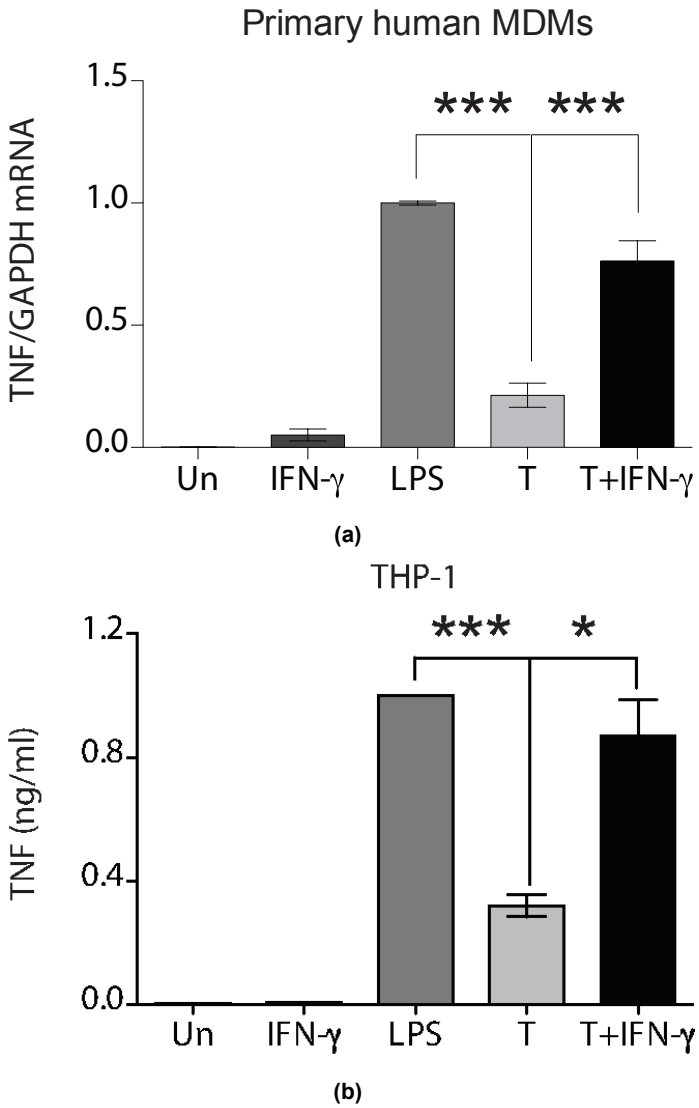


Figure 3.2.1: IFN-γ restores *TNF* gene expression in endotoxin-tolerant monocytes and macrophages. (a) Primary human MDMs were stimulated (experimental design Figure A.1.1), and TNF mRNA levels were measured. Data from 3 separate donors. (b) THP-1 cells were stimulated as in (a) and TNF protein levels in supernatants were measured by ELISA post LPS stimulation (shown relative to LPS values). (*) $p \leq 0.05$ and (***) $p \leq 0.001$.

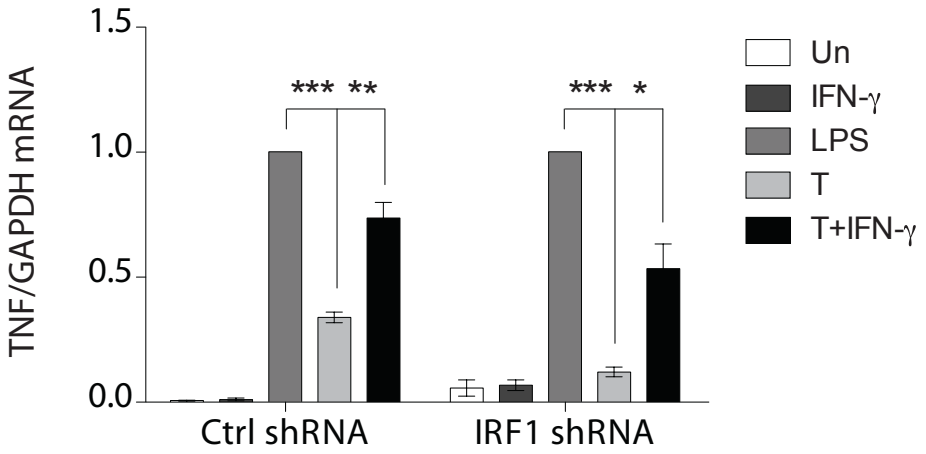


Figure 3.2.2: IFN-γ abrogates endotoxin tolerance in an IRF1-independent manner. THP-1 cells expressing IRF1 and control shRNA were stimulated, and TNF mRNA levels were measured (shown relative to LPS values). (*) $p \leq 0.05$, (**) $p \leq 0.01$, and (***) $p \leq 0.001$.

were treated with IFN-γ for 3h (Figure 2.2.4b), which we concluded as sufficient knock-down of IRF1 protein considering its extreme instability (half-life ~30min) [215]. As expected, IFN-γ priming partially restored *TNF* gene expression in endotoxin-tolerant THP-1 cells expressing control shRNA (Figure 3.2.2). Importantly, partial restoration of *TNF* gene expression also occurred in endotoxin-tolerant THP-1 cells expressing IRF1 shRNA, demonstrating that IFN-γ does not require IRF1 to restore the TNF response during endotoxin tolerance. We hypothesize that two distinct mechanisms are responsible for the effects of IFN-γ in LPS responsive and non-responsive monocytes and macrophages.

3.2.3 IFN-γ promotes chromatin accessibility at the *TNF* promoter in endotoxin-tolerant monocytes.

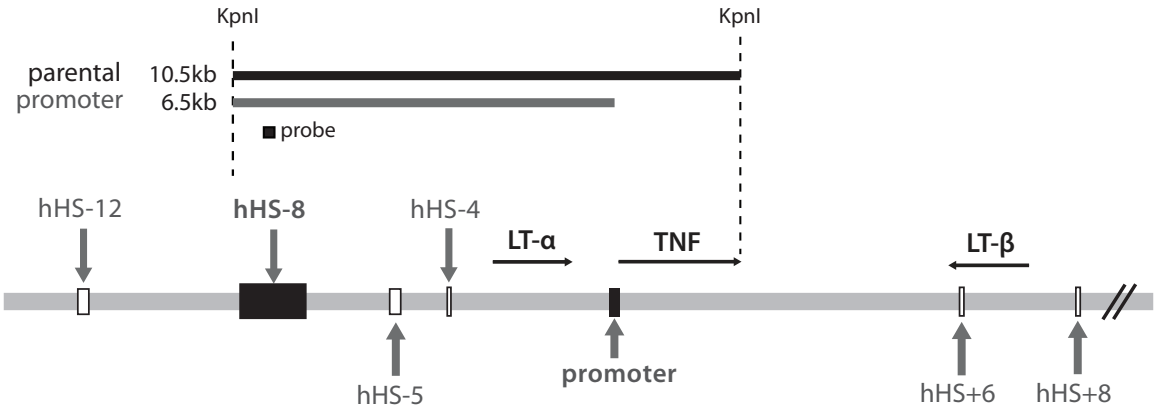
We next examined changes in chromatin accessibility at the *TNF/LT* locus during the induction and abrogation of endotoxin tolerance by performing DNase I hypersensitivity assays (DHAs). We hypothesized that the induction of endotoxin tolerance results in decreased chromatin accessibility at the *TNF* promoter, and that IFN-γ-mediated abrogation of endotoxin tolerance results in the restoration of accessibility. DNA from endotoxin-tolerant and

non-tolerant THP-1 cells stimulated with IFN-γ was partially digested with DNase I. KpnI digestion allowed for visualization of DNase I hypersensitivity at the *TNF* promoter (Figure 3.2.3a). When comparing endotoxin-tolerant and non-tolerant monocytes stimulated with LPS, we observed minimal differences in DNase I hypersensitivity at the *TNF* promoter (Figure 3.2.3b, lane 6 compared to lane 8). We conclude that inhibition of TNF mRNA levels during endotoxin tolerance is not a result of decreased chromatin accessibility at the *TNF* promoter.

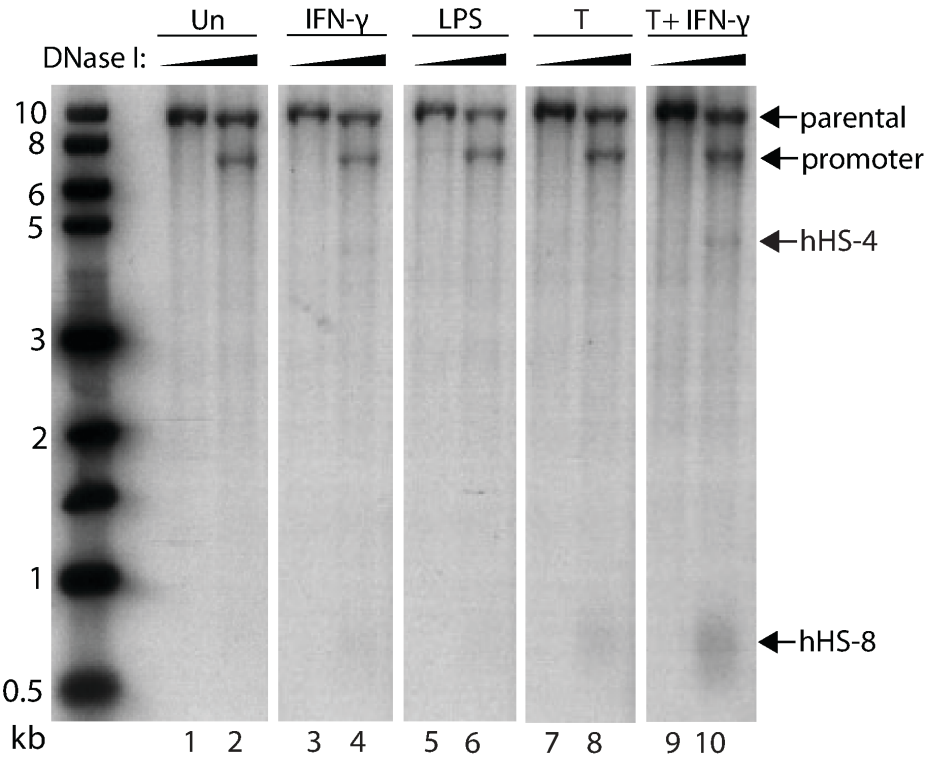
In fact, we observed increased chromatin accessibility, as evidenced by a slight widening of the band representing the *TNF* promoter (Figure 3.2.3b, lane 10). This suggests the appearance of an additional DH site, which may play a role in the mechanism by which IFN-γ priming restores *TNF* gene expression in endotoxin tolerance.

3.2.4 H3K27me3 and H3K27ac levels at the *TNF* promoter predict both the induction and abrogation of endotoxin tolerance in human monocytes and macrophages.

We next investigated histone modifications at the *TNF* promoter during the induction and abrogation of endotoxin tolerance, and identified two modifications that mark LPS responsive and non-responsive monocytes and macrophages. ChIP studies showed significant enrichment of H3K27me3 levels at the *TNF* promoter in endotoxin-tolerant THP-1 cells (Figure 3.2.4a). Conversely, endotoxin-tolerant cells primed by IFN-γ showed no enrichment for H3K27me3 levels at the *TNF* promoter. Results were repeated in primary human MDMs isolated from a representative donor (Figure 3.2.4b). We also observed a complementary response in H3K27ac levels at the *TNF* promoter in response to the induction and the abrogation of endotoxin tolerance. Specifically, IFN-γ priming resulted in a dramatic increase of H3K27ac levels at the *TNF* promoter in endotoxin-tolerant monocytes and macrophages (Figure 3.2.4c, 3.2.4d), which supports the previous finding of increased chromatin accessibility at the *TNF* promoter (Figure 3.2.3b). This data identifies distinct



(a)



(b)

Figure 3.2.3: IFN-γ promotes chromatin accessibility at the *TNF* promoter in endotoxin-tolerant monocytes. (a) Map of the human *TNF/LT* locus. DH sites and positions and directions of transcription of the *TNF*, *LTA*, and *LTB* genes are shown. Positions of the parental KpnI and DNase I digestion products for the DHA are indicated. Fragment representing DNase I accessibility at the *TNF* promoter is 6.5kb in length. (b) IFN-γ increases DNase I cleavage at the *TNF* promoter in endotoxin-tolerant THP-1 cells.

histone modifications at the *TNF* promoter that associate with the induction and abrogation of endotoxin tolerance.

3.2.5 IFN-γ induces the formation of hHS-4 at the *TNF/LT* locus in endotoxin-tolerant human monocytes.

In addition to the *TNF* promoter, we observed chromatin alterations at distal DNA elements within the *TNF/LT* locus during the induction and abrogation of endotoxin tolerance. DNA from endotoxin-tolerant and non-tolerant THP-1 cells stimulated with and without IFN-γ was partially digested with DNase I. ScaI digestion allowed for visualization of both hHS-8 and hHS-4 (Figure 3.2.5a). We detected a strongly enhanced band corresponding to hHS-8 from endotoxin-tolerant cells as compared to non-tolerant cells stimulated with LPS (Figure 3.2.5b, lane 9 compared to lane 12). In addition, increased chromatin accessibility at hHS-8 was sustained in endotoxin-tolerant cells treated with IFN-γ. Past studies have shown that CBP/p300 is recruited to the *TNF* promoter in monocytes and macrophages activated by LPS [189]. We speculate that prolonged exposure and additional challenges of LPS induce continued CBP/p300 recruitment to the *TNF* promoter, thereby promoting DNase I cleavage at hHS-8.

Furthermore, IFN-γ priming in endotoxin-tolerant cells resulted in the formation of the DH site hHS-4 (Figure 3.2.5b, lane 15). This DH site is located ~4kb upstream of the *TNF* TSS and lies within the *LTA* promoter. Although hHS-4 (Figure A.1.2) is not constitutively present in monocytes and macrophages, studies from the Goldfeld lab have shown that hHS-4 is constitutively present in $CD4^+$ T cells (unpublished data). We also observed a slight increase in DNase I cleavage at hHS-4 in non-tolerant monocytes primed by IFN-γ. It is important to note that although non-tolerant and endotoxin-tolerant cells are primed by IFN-γ for the same duration (2h), induction of hHS-4 is significantly stronger in endotoxin-tolerant cells. This suggests that endotoxin tolerance provides an environment that promotes

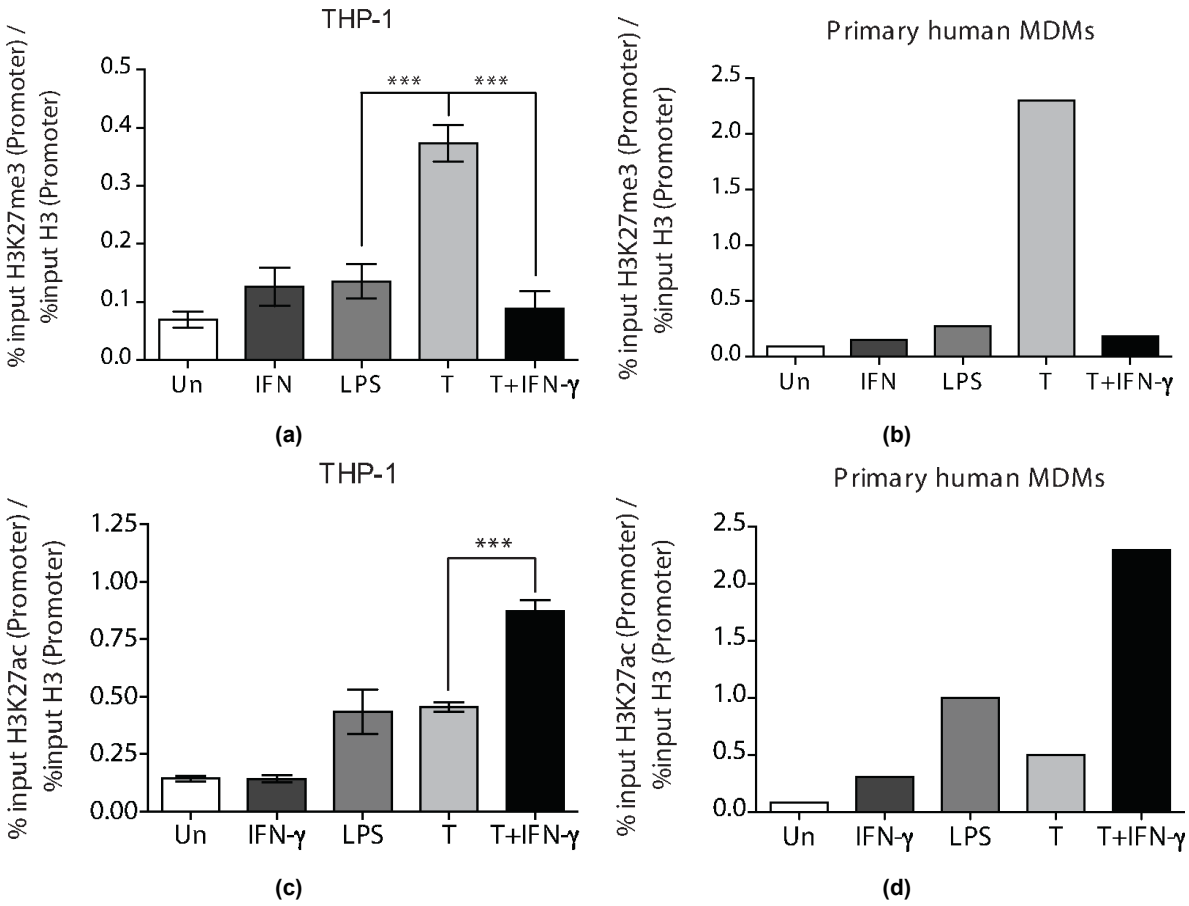
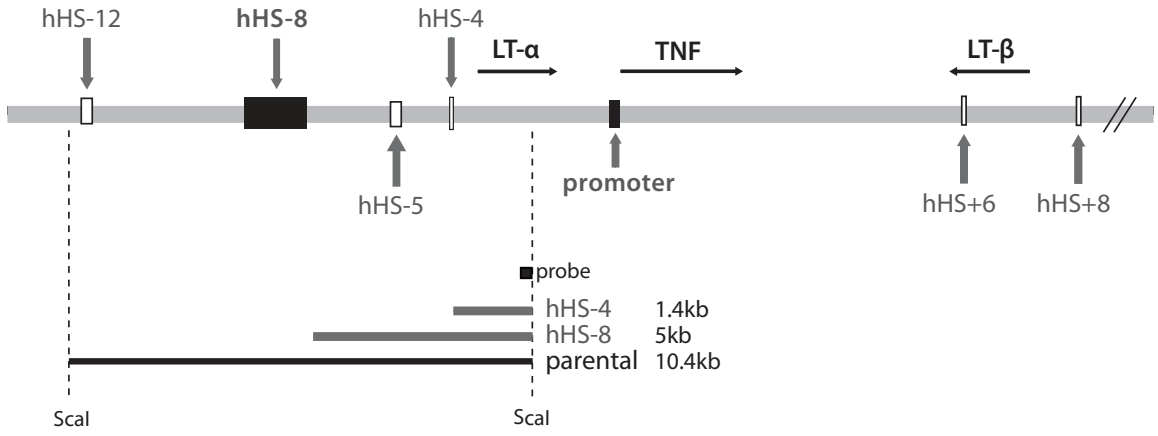
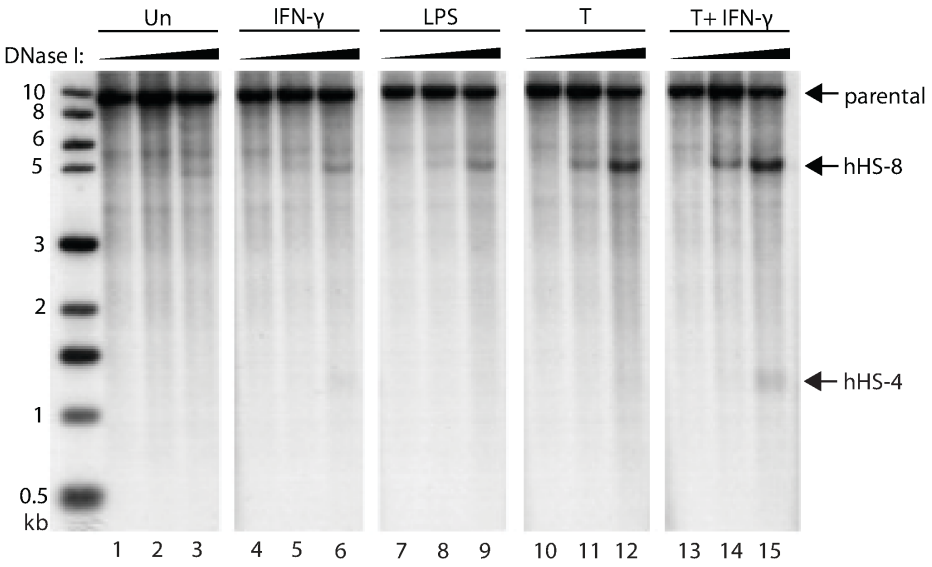


Figure 3.2.4: H3K27me3 and H3K27ac levels at the *TNF* promoter predict both the induction and abrogation of endotoxin tolerance in human monocytes and macrophages. (a) Induction of endotoxin tolerance correlates with an enrichment of H3K27me3 at the *TNF* promoter. ChIP using THP-1 cells and analyzing H3K27me3 prevalence. (b) ChIP as in (a) using primary human MDMs from a representative donor. (c) Abrogation of endotoxin tolerance correlates with an enrichment of H3K27ac at the *TNF* promoter. ChIP using THP-1 cells and analyzing H3K27ac prevalence. (d) ChIP as in (c) using primary human MDMs from a representative donor. (*) $p \leq 0.05$, (**) $p \leq 0.01$, and (***) $p \leq 0.001$.

chromatin accessibility at hHS-4.



(a)



(b)

Figure 3.2.5: IFN-γ induces the formation of hHS-4 at the *TNF/LT* locus in endotoxin-tolerant human monocytes. (a) Map of the human *TNF/LT* locus. DH sites and positions and directions of transcription of the *TNF*, *LTA*, and *LTB* genes are shown. Positions of the parental ScaI and DNase I digestion products for the DHA are indicated. Fragment representing DNase I accessibility at the hHS-8 and hHS-4 is 5kb and 1.4kb in length, respectively. (b) IFN-γ dramatically increases DNase I cleavage at hHS-4 in endotoxin-tolerant THP-1 cells, as compared to non-tolerant THP-1 cells.

3.3 Discussion

Recent studies have highlighted the importance of examining chromatin and its environment when describing endotoxin tolerance and its IFN-γ-mediated abrogation. In this study, we have characterized the *TNF/LT* locus in endotoxin-tolerant monocytes and macrophages and found the *TNF* promoter to be nuclease accessible and enriched for H3K27me3 levels during repressed transcription. We have also found that IFN-γ priming restored *TNF* gene expression in an IRF1-indepenent manner; furthermore, we observed an enrichment of H3K27ac levels at the *TNF* promoter and the appearance of a DH site ~4kb upstream of the *TNF* TSS. These results support the finding that chromatin modifications at both the *TNF* promoter and distal DNA elements are critical for determining mechanisms for the induction of endotoxin tolerance and its IFN-γ-mediated abrogation.

We speculate that *TNF* and *IL6* genes are silenced by different mechanisms, and that mechanisms responsible for transcriptional silencing during endotoxin tolerance vary between genes and their respective loci. One study showed that LPS stimulation in endotoxin-tolerant murine macrophages resulted in decreased nuclease accessibility at the *IL6* promoter, providing evidence for a nucleosome barrier that blocks LPS-induced *IL6* gene expression [236]. Importantly, the authors noted that they did not detect enrichment of the normally repressive histone modification H3K27me3 at the *IL6* promoter. Another study confirmed these results and also demonstrated that priming human monocytes with IFN-γ prevents endotoxin tolerance, evidenced by restored IL-6 mRNA levels and chromatin accessibility at the *IL6* promoter [239]. In contrast, we observed comparable levels of nuclease accessibility at the *TNF* promoter in non-tolerant and endotoxin-tolerant monocytes stimulated with LPS. In addition, we observed an increase in H3K27me3 levels at the *TNF* promoter. Our data suggest that, unlike IL-6, *TNF* gene repression in endotoxin-tolerant monocytes does not involve alterations in chromatin accessibility at the promoter.

What then is the mechanism of *TNF* gene repression during endotoxin tolerance? As stated previously, H3K27me3 has been shown to increase the affinity of the chromodomain of the Drosophila Polycomb protein for histone tails, and this has been implicated in the maintenance of gene silencing [138, 139]. However, mechanisms of Polycomb mediated repression remain unknown. Notably, one study examining the *TNF* promoter in endotoxin-tolerant THP-1 cells observed an enrichment of H3K9me2 levels and an increase in DNA methylation, as compared to non-tolerant THP-1 cells stimulated with LPS [240]. They also show recruitment of both the H3K9 histone methyltransferase G9a and the DNA methyltransferase Dnmt3a/b to the *TNF* promoter during endotoxin tolerance. Unfortunately, we were unable to repeat the H3K9me2 findings. Future experiments will have to explore the functional role of histone and DNA methylation in *TNF* gene repression during endotoxin tolerance.

We found chromatin modifications during IFN-γ-mediated abrogation of *TNF* gene repression to be similar to that of *IL6*; specifically, we observed an increase in nuclease accessibility and an enrichment of H3K27ac levels at the *TNF* promoter. Notably, past studies have prevented induction of endotoxin tolerance by pretreating cells with IFN-γ before the initial LPS challenge [239] whereas our work investigates the ability of IFN-γ to abrogate endotoxin tolerance after it has been induced. We find our work to be clinically more relevant when thinking of potential therapeutic targets for sepsis patients whose monocytes and macrophages have already entered an immunosuppressed state.

In addition, we have shown that IRF1 does not play a critical role in the ability of IFN-γ to restore *TNF* gene expression in endotoxin-tolerant cells, which suggests that IFN-γ priming employs distinct mechanisms for enhancing *TNF* transcription in resting cells as compared to endotoxin-tolerant cells. This we found unsurprising considering the fact that the chromatin environment of the *TNF/LT* locus is markedly different between resting and endotoxin-tolerant cells; thus, IFN-γ stimulation must employ distinct mechanisms to pro-

mote a chromatin environment that favors enhanced *TNF* gene expression. It will be important to identify transcription factors and chromatin remodeling enzymes essential for IFN-γ-mediated abrogation of endotoxin tolerance; STAT1 may play an important role considering its activation by IFN-γ signaling.

Finally, identification of hHS-4 as an IFN-γ-inducible DH site in endotoxin-tolerant monocytes suggests its role as a distal regulatory element. Although hHS-4 lies within or near the *LTA* promoter [242], preliminary experiments have shown that IFN-γ does not induce LT-α expression in endotoxin-tolerant monocytes or macrophages (data not shown). Notably, hHS-4 is constitutively present in $CD4^+$ T cells; thus, it may function as both a promoter in lymphocytes and an IFN-γ-inducible regulatory element in endotoxin-tolerant monocytes and macrophages. Future experiments will have to explore the functional role of hHS-4, particularly as an enhancer or insulator.

3.4 Materials and Methods

Cell culture and stimulations

THP-1 cells were maintained in RPMI-1640 supplemented with 10% FBS. For primary human MDMs, enriched populations of human monocytes were isolated from healthy human donor buffy coats using a $CD14^+$ positive selection kit (Stemcell). MDMs were obtained after 6d of culture in RPMI-1640 medium supplemented with 5% human serum AB (Gemcell) and GM-CSF (50ng/ml; Peprotech). Cells were treated with IFN-γ (100ng/ml; R&D) and LPS (100ng/ml; Sigma E. coli O111:B4).

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from cells with a QuickRNA Mini kit (Zymo) and treated with Turbo DNA-free kit (Invitrogen). cDNA was synthesized from total RNA with M-MLV reverse transcriptase (Invitrogen) and 20-residue oligo (dT) (Invitrogen). TNF mRNA levels

were measured by the change-in-threshold (ΔΔCt) method based on quantitative real-time PCR in an iCycler iQ (Bio-rad) with SyberGreen Master Mix (Invitrogen) and primers recognizing exon 4 and exon 3 of the human *TNF* gene and the human *GapdH* gene (Table 2.4.1).

DNase I Hypersensitivity Assay (DHA)

DHAs were performed using THP-1 cells. Cells were harvested, washed with PBS, and resuspended in RSB buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, and 3mM MgCl2). Cells were lysed with lysis buffer (0.5% NP-40 in 1x RSB buffer) on ice for 5min. Resuspended nuclei in RSB buffer were treated with DNase I (40ng/ul) at 37C for 5min. DNase I activity was quenched upon addition of stop solution (0.6M NaCl, 20mM Tris-HCl pH 8.0, 10mM EDTA, and 1% SDS). Samples were treated with Proteinase K at 56C O/N. For analysis of hHS-4, DNA was digested with either ScaI or KpnI and analyzed by Southern blotting using a radiolabeled P^{32} probe corresponding to the coding region of *LTA* or hHS-8, respectively. 10ug of DNA was used for each lane.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with anti-H3K27me3 (C36B11, Cell Signaling), anti-H3K27ac (Active Motif), and anti-H3 (Abcam). THP-1 cells and primary human MDMs were treated (IFN-γ 100ng/ml, LPS 100ng/ml), fixed with 10% formaldehyde for 15min, treated with 2.5M glycine for 5min, harvested, washed with PBS, lysed with 0.25% Triton X-100 and 0.5% NP-40 for 5min, centrifuged at 1200RPM for 10min, resuspended in 1% SDS lysis buffer, and sonicated 5min for 4 cycles in a Biorupter. Sonicated DNA was set up for immunoprecipitation O/N and DNA-protein complexes were recovered by adding Protein A/G Plus Agarose Beads (Thermo Scientific) for 3h. Samples were washed 6 times with 1ml of wash buffer and treated with proteinase K at 65C O/N. Samples were treated

with phenol/chloroform before O/N ethanol precipitation. DNA fragments for H3K27me3 and H3K27ac analysis were analyzed by quantitative real-time PCR using Jumpstart™ Taq ReadyMix™ For Quantitative PCR (Sigma) and a primer/probe set for the *TNF* promoter (Table 2.4.1). H3K27me3 and H3K27ac percent input values were normalized to H3 percent input values.

Short hairpin RNA

The lentiviral plasmid PLKO.1 expressing shRNA targeting human IRF1 was purchased from the RNAi Consortium (TRC) Lentiviral shRNA library (Thermo Scientific). Clone TRCN0000014668 with a target sequence of CGTGTGGATCTTGCCACATTT was validated in our laboratory. Control shRNA encodes a scrambled sequence. Lentiviruses encoding shRNA sequences were generated by transfecting the packaging cell line HEK-293T with the shRNA-encoding pLOK.1 plasmids in combination with the packaging plasmid psPAX2 and the envelope plasmid pMD2.G using Effectene transfection reagent (Qiagen). Supernatants were collected 48h post-transfection, clarified by centrifugation, and stored at -80C. THP-1 cells were transduced with the lentiviral particles by culturing the cells with supernatants from the virus-producing cells in the presence of 8ug/ml polybrene (Millipore) and spinoculation for 2h at 2000RPM. Successfully transduced cells were selected and expanded by treatment with 0.8ug/ml puromycin.

Chapter 4

Concluding Remarks

The overarching goal of this thesis project was to use the *TNF/LT* locus as a model system to identify and dissect mechanisms of chromatin remodeling that control classical macrophage activation and endotoxin tolerance. In doing so, we have provided a mechanistic explanation of how IFN-γ poises the *TNF/LT* locus for enhanced *TNF* transcription upon LPS stimulation, while at the same time providing potential targets for selective manipulation of TNF expression in primed macrophages. We have also characterized the chromatin environment at the *TNF/LT* locus during the induction and IFN-γ-mediated abrogation of endotoxin tolerance. More generally, we have examined long-range distal DNA elements of the *TNF/LT* locus and demonstrated the importance of examining DNA elements beyond the promoter when investigating the elaborate nature of inflammatory responses and developing novel therapeutics for inflammatory diseases.

Importantly, distal DNA elements, particularly enhancers, are thought to be of primary importance when determining cell type-specific gene expression [122]. This is supported by the finding that enhancers have been observed to be largely cell selective, to be marked with cell type-specific histone modification patterns, and to actively function in a cell type-specific manner. Promoters, in contrast, have been observed to appear and function in a similar manner across cell types. In the *TNF/LT* locus, hHS-8 is present in the primary

producers of TNF (i.e., monocytes, macrophages, and T cells); it is of interest to examine whether hHS-8 is present in other cells that are not primary producers of TNF.

As stated previously, studies using mice with cell type-specific deletions of the *TNF* gene have suggested that novel therapies for autoimmune diseases that specifically target myeloid-derived TNF would be preferable in latently infected TB patients [39]. In the case of our proposed model for IFN-γ priming, hHS-8 and its chromatin environment may provide potential targets for selective manipulation of TNF expression in monocytes/macrophages as compared to T cells. Although HSS-9 (murine hHS-8) was shown to function as an inducible enhancer in murine T cells [177], we hypothesize that transcription factors, chromatin-remodeling enzymes, and patterns of histone modifications will differ at hHS-8 between T cells and monocytes/macrophages. This is similarly seen at the *TNF* promoter when looking at enhanceosome formation in T cells and monocytes/macrophages [175].

In addition to demonstrating a high degree of cell type-specificity, distal DNA elements can be inducible and inducer-specific. Thus, it is critical to search for distal DNA elements in model systems that represent disease states. For example, in the *TNF/LT* locus, hHS-4 has been shown to be cell type-specific between resting primary monocytes and $CD4^+$ T cells, and we have now shown hHS-4 to be inducer-specific within endotoxin-tolerant monocytes and macrophages. This finding demonstrates that selective modulation of individual genes of the inflammatory response requires a thorough understanding of inducible chromatin modifications for both the promoter and distal regulatory elements in different settings of pathology. In the case of TNF dysregulation, future experiments would ideally examine chromatin modifications at the *TNF/LT* locus in resting and IFN-γ-treated monocytes isolated from sepsis patients.

Lastly, we have identified hHS-8 and hHS-4 by DNase I hypersensitivity assays (DHAs) followed by Southern blotting. This method is feasible for identifying distal DNA elements $\leq$50kb from the target promoter; however, regulatory elements have been described as tens

to hundreds of kilobases away from their target promoter. Indeed, identifying distal DNA elements with respect to target genes can be quite challenging. Often times, distal DNA elements are assigned the closest gene as their target gene; unfortunately, there has been no report to date on the rate of false positives for this strategy. A recent study has applied chromosome conformation capture (3C) assay technology to identify interactions between transcription start sites (TSSs) and previously identified distal DNA elements throughout the human genome [174]. Considering the fact that enhancers are often found to be in close proximity with their target promoters, this method is thought to hold promise in identifying a percentage of target promoters. Ultimately, a greater understanding of how enhancers and other types of regulatory elements mechanistically function is required for high throughput methods of classifying regulatory elements with their target promoters.

Appendix A

Supplemental Figures

A.1 Supplemental Figures

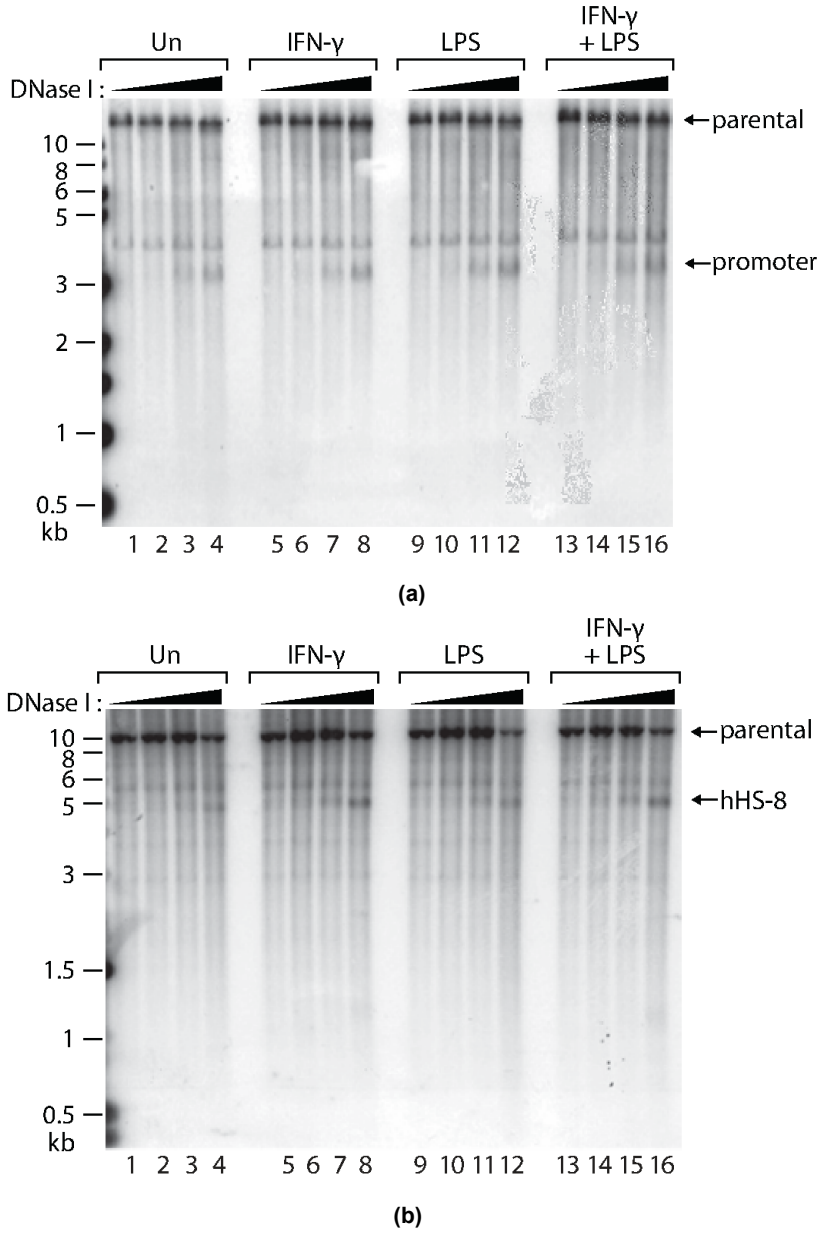


Figure A.0.1: IFN-γ promotes chromatin accessibility at hHS-8 in the *TNF/LT* locus. IFN-γ does not affect DNase I cleavage at the *TNF* promoter. DHA using THP-1 cells. DNase I digestion products for the DHAs are indicated; fragment representing DNase I accessibility at the *TNF* promoter and hHS-8 is $\sim$3kb and $\sim$5kb in length, respectively. (c) IFN-γ strongly increases DNase I cleavage at hHS-8.

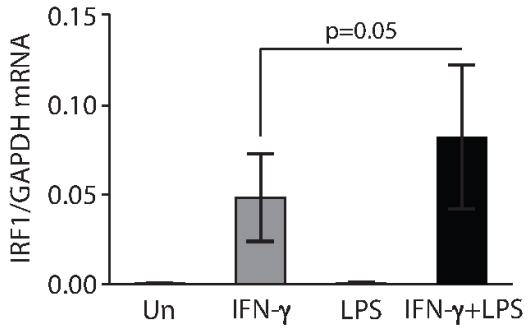


Figure A.0.2: THP-1 cells were stimulated, and IRF1 mRNA levels were measured.

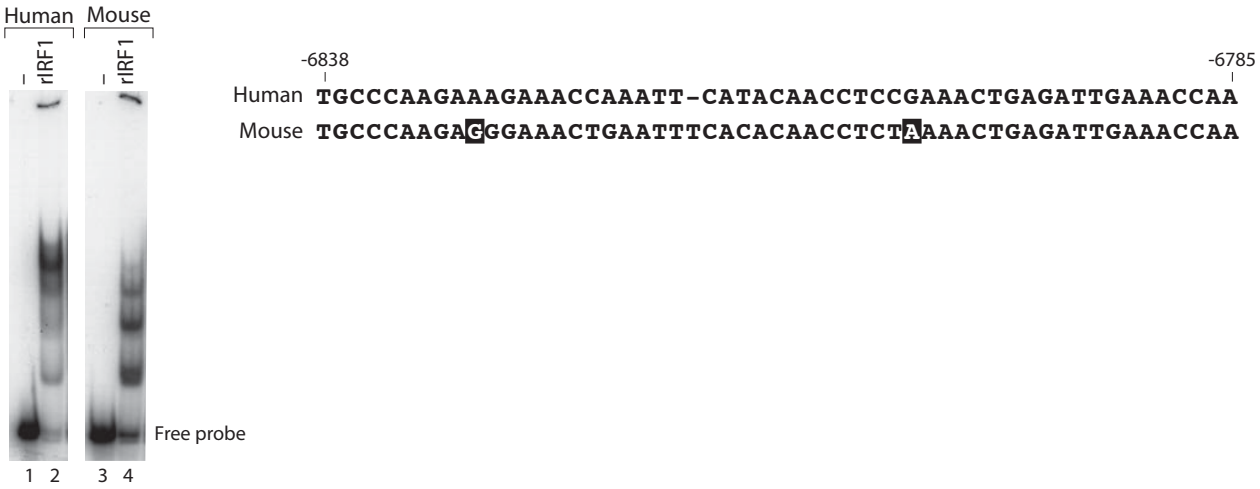


Figure A.0.3: rIRF1 binds to hHS-8 in both the human and mouse *TNF/LT* locus. EMSA was performed with rIRF1 and human and mouse radiolabeled P^{32} oligonucleotides of sequences for positions -6838 to -6785.

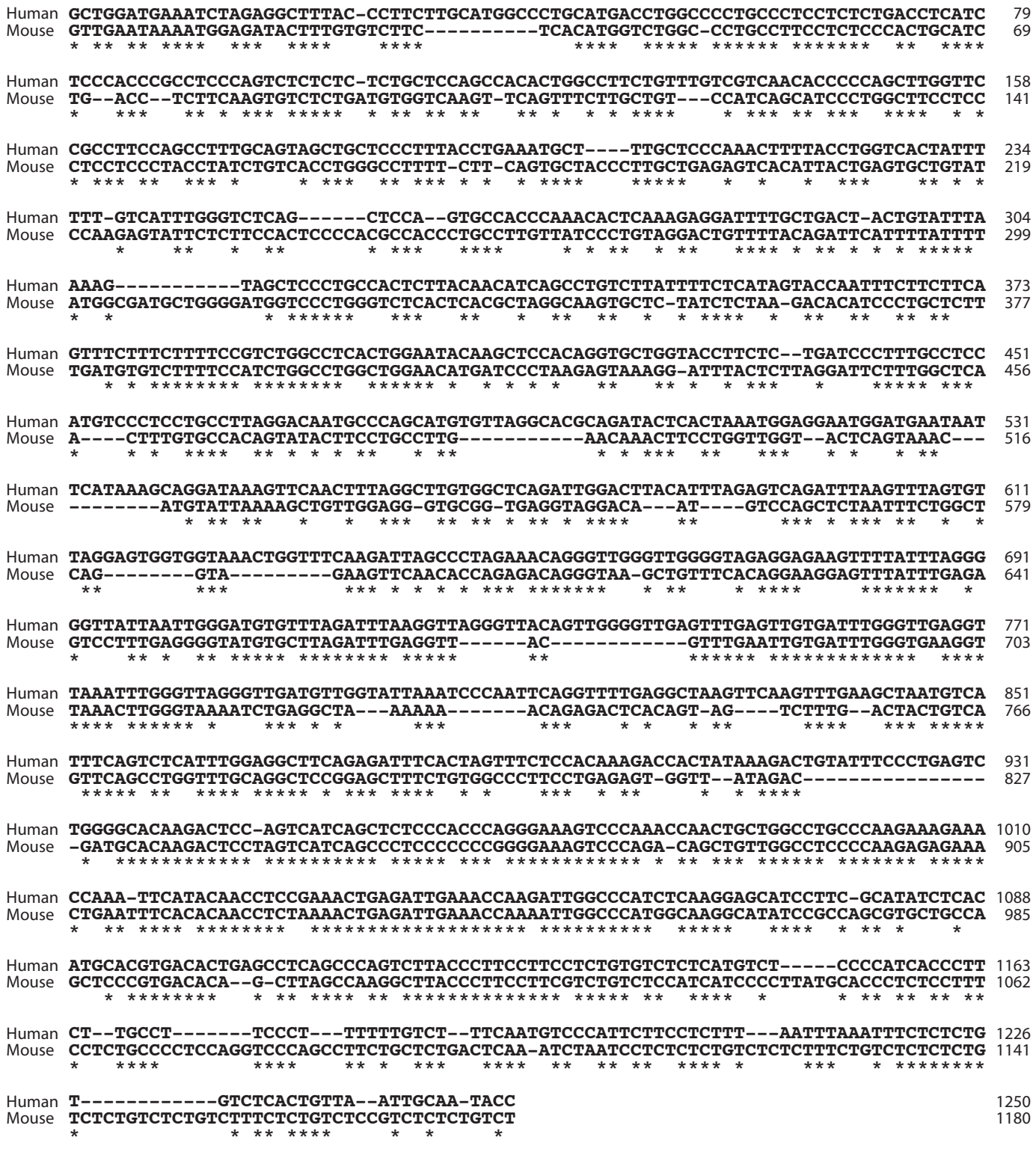


Figure A.0.4: hHS-8 is highly conserved between human and mouse. Alignment of human and mouse sequences of hHS-8 (∼1250bp).

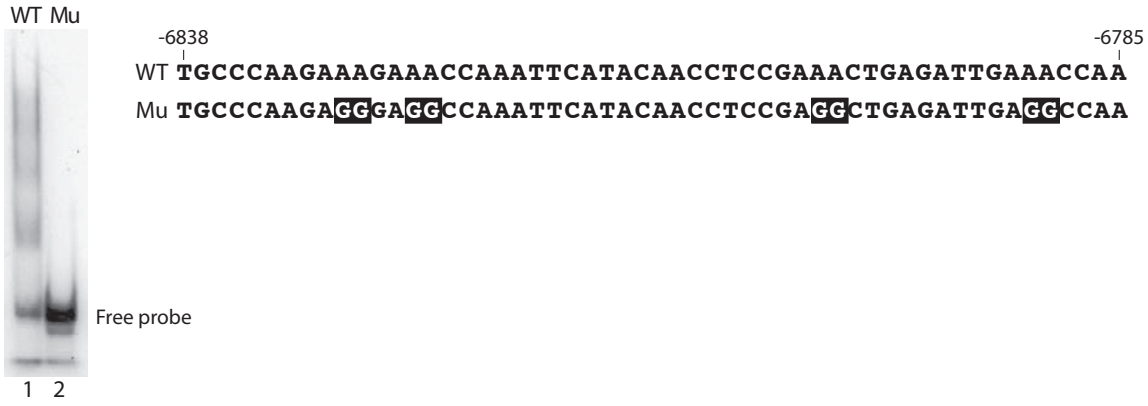


Figure A.0.5: Nucleotide changes in the critical -GAAA- motifs disrupt rIRF1 binding. EMSA was performed with rIRF1 and wildtype (wt) and mutant (mu) radiolabeled P^{32} oligonucleotides (sequences for positions -6838 to -6785).

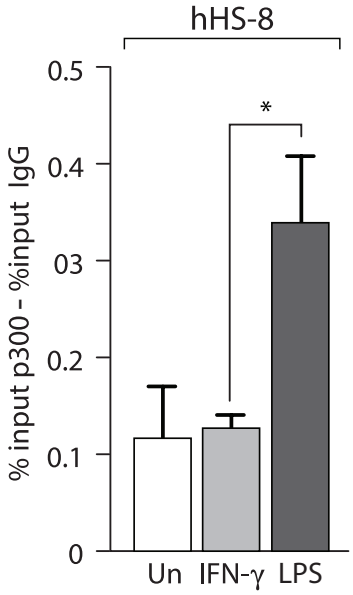


Figure A.0.6: p300 is recruited to hHS-8 upon LPS stimulation. ChIP using primary human MDMs and analyzing p300 recruitment to hHS-8. Stimulations were IFN-γ alone for 3h, LPS alone for 1h, and both IFN-γ and LPS (IFN-γ pretreatment for 2h followed by LPS treatment for 1h). (*) $p \leq 0.05$. Data from 3 separate donors.

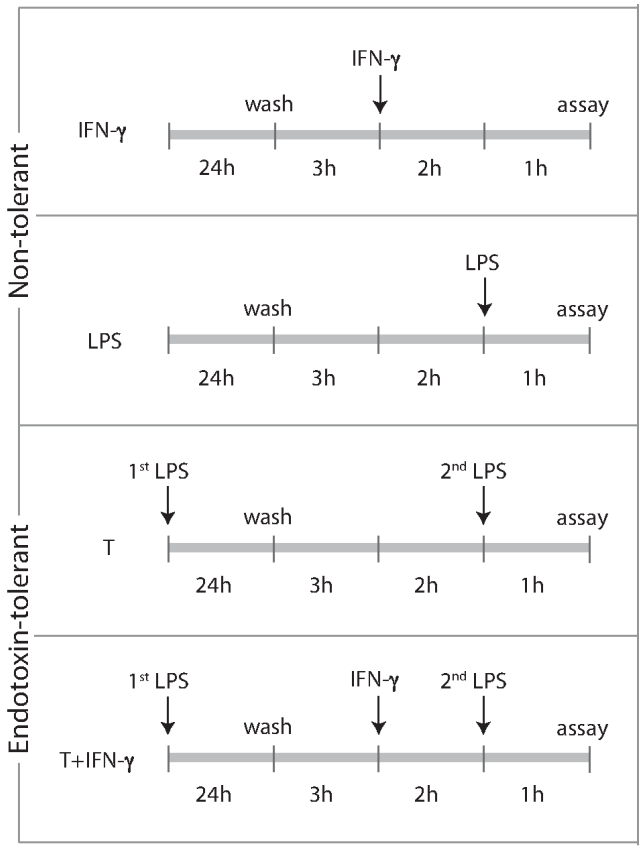


Figure A.1.1: Experimental design of endotoxin tolerance induction and IFN-γ-mediated abrogation of endotoxin tolerance.

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Human  CCTCACCCATGTGGAATTCTG--------AAACTTCCTTTGTAGAAAACTTTGGAAGGTG 52
Mouse  -ATAAATGATGAGCAAATTTATATACAACAAAGTTATCTGTCATAAATATATGTAAGAAG 59
         * *   *** * ** * *         *** **   *   * ***  * ** ***  *

Human  TCTGCCACATTGATCCTGGAATGTGTGTTTATTTGGGGTTATATAAATCTGTTCTGTG-- 110
Mouse  TA------ATAAGTCCTTTG-TGTGTGTTTATGTGTGGTTATATGTATGTGTGCTGTGTG 112
       *       **   ****    *********** ** ********  ** *** *****

Human  --GAAGCCACCTGAAGTCA--- 127
Mouse  TGGAAGCCA---GAGGACAACC 131
         *******   ** * **
```

Figure A.1.2: hHS-4 is highly conserved between human and mouse. Alignment of human and mouse sequences of hHS-4 (~120bp).

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